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TITLE: Advanced Developement of Leishmania tropical Skin Test Antigen

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14. ABSTRACT
Leishmania tropica Skin Test Antigen (LtSTA) is lysate of microfluidized L. tropica promastigotes. The product is manufactured at a protein concentration of 0.5 mg/mL. Lot consistency is established by densitometry readings of SDS-PAGE gels and delayed-type skin tests in sensitized guinea pigs. The dominant proteins in the promastigote lysate are found at 8 kDa, 20kDa, 30kDa, and 56-58 kDa; of these, the 8 kDa and 56-58 kDa entities are most prominent. The 8 kDa component is believed to be ubiquitin consisting of several proteases. The 56-58 kDa component was shown to contain metalloproteases, trypanothione reductase and dihydrolipamide dehydrogenase by NanoLC-ESI-MS/MS peptide sequencing technology. By densitometry, the relative amounts of the five major proteins in the lysate were as follows: 8 kDa (10.1-31%), 20 kDa (3.9-7.8 %), 30 kDa (3.4-9.2%), 56 kDa (28.9-40.1%), 58 kDa (31.3-40.1). Based on a validated assay using sensitized guinea pigs, lotscontaining 0.34 mg/mL to 0.61 mg/mL of protein can be considered equipotent to 0.5 mg/mL.

15. SUBJECT TERMS
LtSTA = Leishmania tropica Skin test Antigen

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ANNUAL REPORT

June 18, 2010 – August 31, 2011
Allermed Laboratories, Inc.
Contract W81XWH-09-C-0138

SECTION 1. INTRODUCTION

This report covers work performed between June 18, 2010 and August 31, 2011 on a Leishmania Skin test (LtSTA) made from the promastigotes of *Leishmania tropica*. During the last reporting period between June 18, 2009 and June 17, 2010, Allermed had completed a phase IIB study involving the sensitizing capacity of three different doses of the product. The results of this study were submitted to the FDA for review with a request for a Type B Meeting to discuss the pathway forward leading to the design and implementation of a phase III protocol. As a result of this request, the FDA asked for additional information on the composition of the skin test antigen and the indicated use of the product.

SECTION 2. BACKGROUND

Under contract W81XWH-09-C-0138 Allermed has performed work on phase II development of LtSTA. This work includes the validation of manufacturing and testing procedures and the characterization of major and minor components in the drug substance and drug product prior to advancing the product to phase III development.

SECTION 3. BODY OF REPORT

3.1. **Statement of Work**

The focus of work during the current reporting period was directed to reporting the current status of BB-IND 1182 and responding to two letters that were received from the FDA concerning issues that should be addressed before implementing a phase III clinical trial.

In a letter dated May 7, 2010 (Attachment 1) the FDA requested information about the methods used in the manufacturing and testing of the product, including validation of these procedures. Between May 7, 2010 and November 10, 2010, Allermed completed the required work and submitted a written reply to the FDA on November 10, 2010. In addition, the Annual Report for the IND was sent to the FDA on December 10, 2010 (Attachment 2).

On March 17, 2011, a second letter was received from the FDA requesting additional information relating to the indicated use and characterization of the product. Between March 17, 2011 and August 29, 2011 this work was performed. It involved the characterization of the major and minor components of LtSTA, as well as the validation of key assays used in manufacturing the product. The results of this work were reported to the FDA on August 29, 2011 (Attachment 3). Attachments 1-3, as well as the Quarterly Reports included as Attachment 4, detail the work that has been performed during the current period.

3.2 **Key Accomplishments**

Based on the results of a phase IIB clinical trial in which 15 ug, 30 ug and 50 ug doses of Lysate protein were evaluated for false positive reactions and sensitizing capacity, it was determined that phase III development of the LtSTA would be based on a dose of 50 ug. This decision was made in consultation with representatives of USAMMDA and Dr. Afif Ben Salah of the Pasteur Institute in Tunis, Tunisia. The primary factors that were considered in selecting the 50 ug dose were (1) a 50 ug dose elicited positive skin tests in persons with a history of cutaneous leishmaniasis that were skin test negative to a 30 ug dose, and (2) sensitization in *Leishmania* naïve subjects occurred to both the 30 ug and 50 ug doses after repeat skin testing; this finding indicated that neither dose was non-sensitizing and, therefore, future work should be done with a 50 ug dose, because of its increased sensitivity mentioned in (1) above.

Until a specific dose was selected for the future development of LtSTA, it was not possible to validate processes according to the chemistry, manufacturing and control aspects of product development. Therefore, the key accomplishments that were realized during this reporting period were related to these activities for LtSTA made at a concentration of 0.5 mg/mL.

3.2.1 Manufactured two stability lots at 0.5 mg/mL and placed on an accelerated and real-time, long-term stability protocol. Final product lot LtSTA01 and Lot StSTA02 were placed on accelerated stability conditions at 23-27 C and 35-40 C and real-time stability conditions at 2-8 C. The testing that has been completed to date, confirms the stability of the products for 3 and 6 month time points under accelerated conditions. Testing of the lots stored at 2-8 C will be on going for the next 3-5 years.

3.2.2 Manufactured two internal reference standards at 0.5 mg/mL Determined that storage conditions at 2-8 C were superior to storage at -20 C and -80 C and storage after lyophilization.

3.2.3 Characterized drug substance and drug product by SDS-PAGE, densitometry, and mass spectrometry.

3.2.4 Sequenced and compared dominant proteins in promastigote lysate with other *Leishmania* species, Trypanosoma, misc. biological materials and humans.

3.2.5 Validated key steps in the manufacturing process, including microfluidization, aseptic filtration, mixing and holding times, protein determination by the Ninhydrin Method and potency using a parallel bioassay in sensitized hairless guinea pigs.

3.2.6 Submitted written responses to FDA letters dated May 7, 2010 and March 17, 2011.

3.2.7 Submitted Annual Report for BB-IND 11822

3.2.8 Submitted Quarterly Reports for contract W81XWH-09-C-0138

3.2.9 Held teleconference meetings with representatives of USAMMDA

SECTION 4. REPORTABLE OUTCOMES

4.1.1 Lot Size and Numbering System of 0.5 mg/mL LtSTA

During this reporting period, the process that had been used to manufacture the drug product (LtSTA) from the drug substance (lysate) was changed. To provide drug substance to make enough bulk drug product for approximately 5,000 vials of LtSTA, it was necessary to combine two lots of lysate before diluting the material to a Ninhydrin protein concentration of 0.5 mg/ml. Prior to this change, all lots of LtSTA were identified by the lot number assigned to the drug substance. However, this method of lot number assignment was not used after LtSTA was made from two lots of lysate. The lot numbering system was simplified to LtSTA with the suffix 01, 02, 03, etc.

4.1.2 Effects of temperature on 0.5 mg/mL LtSTA

Over the course of development, several different reference preparations have been evaluated for reproducibility and stability. These references have included freeze/thaw preparations of promastigotes, lysate of promastigotes, and LtSTA prepared at various protein concentrations. In addition, the effects of stress conditions such as heating and freezing on candidate references have been investigated. Once it was determined that LtSTA manufactured at 0.5 mg/mL of Ninhydrin protein would be used in all future clinical trials, references containing 0.5 mg/ml were manufactured and stored under different conditions to evaluate stability.

Two lots of 0.5 mg/ml reference product (LtLRS01 and LtSRS02) were prepared. The reference product was filled in final containers and underwent complete quality assurance testing following the same procedures that are used in the manufacture of stability lots. These reference lots, LtSTA01 and LtSTA02 were placed at 2-8 C and -80 C and evaluated by ELISA, SDS-PAGE and relative potency in sensitized guinea pigs. Reference lot LtLRS01 was heated at 90 C for 10, 30 and 60 minutes to determine the effects of elevated temperature on the stability of the material. The results of this work are reported in Table 1 and summarized as follows:

a. Storage at -80 C destroys the ability of product to bind with anti-L. tropica rabbit antiserum by ELIZA. The same inability to bind with antiserum was observed in lyophilized product. However, product stored at 2-8 C retained the ability to combine with antibody in the same assay.

b. No reproducible differences were observed by SDS-PAGE in the banding of product stored at 2-8 C and product stored at -80 C.

c. Product stored at 2-8 C elicited larger induration responses in sensitized guinea pigs than the same material stored at -80 C.

d. Heating product at 95 C for 60 minutes removed the 67 KDa band in SDS-PAGE gels. This band in unheated product disappeared after several months when stored at 2-8 C.

e. Loss of potency as measured by induration in sensitized guinea pigs was observed in product heated at 95 C for 60 minutes.

TABLE 1.
SUmmary of SDS-PAGE, ELISA, and Potency Experiments on Leishmania Drug Product

Drug Product	Storage Temp	Results ELISA minimum acceptable S:N = 2.0	Results SDS-PAGE	Results Potency Testing Average Induration for 50μg/0.1mL Dose
LtLRS01	-80°C	Heating at 95°C for: 10, 30, & 60 minutes: S:N = 1.2	Heating (95°C for 60 min): Loss of banding at 67 kDa The unheated sample at -80°C showed strong bands at 8 and 30 kDa, weak band at 67 kDa, and a very weak band at 30 kDa	LtLRS01 at 2-8°C=12mm LtLRS01 at 95°C=10mm LtLRS02 at 2-8°C =13mm
LtLRS02	2-8°C	S:N = 3.2	Strong bands at 8, 30, and 56 kDa, weak band at 67 kDa	11.1mm (F)
LtLRS02	-80°C	S:N = 1.6	Strong bands at 8, and 56 kDa, weaker band at 67 kDa, very weak band at 30 kDa	8.9mm
LtSTA01	2-8°C	S:N = 4.3	Strong bands at 8 and 56 kDa. Weaker bands at 10, 20, and 30 kDa, very weak band at 67 kDa	10.7mm
LtSTA01	-80°C	S:N = 2.0	Same as 2-8°C except for a darker band at 67 kDa	ND
LtSTA02	2-8°C	N/A	Strong bands at 8 and 56 kDa, weaker bands at 10, 20, 30, and 67 kDa	10.0mm (F)
LtSTA02	-80°C	N/A	Same as 2-8°C	ND

4.1.3 Major and Minor Components of LtSTA

In characterizing the drug substance and the final drug product the dominant proteins that are present in LtSTA were indentified using SDS-PAGE, scanning densitometry and mass spectrometry. The presence of glycoproteins and lipoproteins in the crude lysate was investigated using staining procedures that are specific for these compounds. The following information is an overview of the information contained in Allermed's reply to FDA's letter dated March 17, 2011. Please see Allermed's submission dated August 29, 2011 with attachments for more detailed information regarding characterization.

In the SDS-PAGE studies reported in our IND application, polyacrylamide gels were stained with silver nitrate. Using silver nitrate, bands were observed at 70 kDa, 51 kDa, 25 kDa, 20 kDa and 8 kDa. More recently, using Coomassie Blue Stain, bands at 67 kDa, 58 kDa, 30 kDa, 20 kDa and 8 kDa have been observed. The band at 67 kDa is visible in recently manufactured lots

of LtSTA, but disappears within months during storage at 1-5 °C. In comparing gels stained with silver nitrate and Coomassie Blue, the (70 kDa and 67 kDa bands), the (51 KDa and 56 kDa bands), and the (25 kDa and 30 kDa bands) appear to represent the same components. One published study (2) supports the immunogenicity of the 58 kDa component which is present as a major component in LtSTA as shown by scanning densitometry.

Based on the protein bands present in SDS-PAGE gels stained with Coomassie Blue, the proteins identified at 8 kDa, 20 kDa, 30 kDa, 56-58 kDa and 67 kDa were analyzed by NanoLC-ESI-MS/MS peptide sequencing technology. The protein in the 8 kDa band was identified as ubiquitin which is consistent with the fact that several proteases were present in the sample. The 56-58 kDa bands contained several proteins of similar molecular weight. Using *L. major* as a reference, the compounds identified in the 20 kDa, 30 kDa, 56-58 kDa were as follows: 20 kDa (threonine peptidase and iron superoxide dismutase), 30 kDa (aldolase and cathepsn L-like protease), and 56-58 kDa (metalloproteases, trypanothione reductase and dihydrolipoamide dehydrogenase).

The relative quantities of the 8 kDa, 20 kDa, 30 kDa, 58 kDa and 67 KDa components were determined by scanning densitometry. Six bands were detected in gels stained with Coomassie Blue. Six lots of drug substance and two lots of drug product were evaluated. The gel image revealed conspicuous bands at 8 kDa and 56-58 kDa in both the drug substance and drug product. Bands at 20 kDa and 30 kDa were less conspicuous in both the drug substance and drug product, and a band at 67 kDa was only visible in two lots of drug substance which had been recently manufactured relative to the other four lots of drug substance. The 67 kDa band was not visible in the drug product. In the drug substance the 56 and 58 kDa bands accounted for 60-70% of the protein in each lot; these bands were detected as a single band in the drug product accounting for 40 % of the protein in the samples. Differences identified by the densitometer in the intensity and relative percentages of each band measured in the drug substance and drug product were believed to be due to the concentrations of protein present. The drug substance contained 3-5 mg/mL, whereas the drug product contained 1.5 mg/mL (note: it was necessary to concentrate the 0.5 mg/mL drug product 3X in order to visualize the bands with Coomassie Blue stain). With only one band detected at the 56-58 kDa location in the more dilute drug product, the relative percentage of each band in the drug product changed, which is believed to account for the increase in the percentage of material at 8 kDa. It was not possible to establish specifications for the relative quantities of the major protein components in the final product, due to the faintness of the protein bands when stained with Coomassie Blue. For this reason, specifications have been established for the drug substance which can be stained with Coomassie Blue and analyzed by scanning major proteins in the drug substance. These specifications are as follows: 8 kDa (10.1 – 31%), 20 kDa (3.9-7.8%), 30 kDa (3.4-9.2%), 56 kDa (28.9-40.1%), 58 kDa (31.3-40.1%). A range for the 67 kDa band was not established, because of the transient nature of the band.

Final product will be stained using silver nitrate to verify the bands observed in the drug substance are present in the final product. At the drug product concentration of 0.5 mg/mL, the 56 kDa and 58 kDa appear as one band on sliver stained gels. This was also observed using Coomassie Blue Stain with product containing 1.5 mg/ml. Final product must contain the 8 kDa, 20 kDa, 30 kDa, and 56 kDa bands by silver stain to be acceptable. Other bands that are present in silver stained gels will not be monitored

Evaluating the presence of glycoproteins and lipoproteins in the crude lysate has been accomplished by specific staining procedures. However, in-depth studies of proteins modified with these components are very complex and, in our opinion, go beyond the scope of this project. We have tested LtSTA with gel-based glycoprotein and lipoprotein assays and have not found these compounds to be present in LtSTA.

Phenolated and non-phenolated LtSTA were analyzed by SDS-PAGE , densitometry and mass spectrometry. The proteins present in the 8 kDa, 20 kDa, 30 kDa, 56/58 kDa and 67 kDa bands were determined. No major differences were detected in the phenolated and non-phenolated samples by these procedures. The phenolated product contained all of the proteins that were present in the non-phenolated product, as well as two additional proteases (LmjF.33.1610 and LmjF.05.0960). Over all, the phenol preservative in LtSTA did not appear to have a negative effect on the lysate.

4.1.4 Correlation of Allermed's Data with Published Data

From the published literature, a variety of compounds are present on the surface and internal structure of the *Leishmania* promastigote which are potentially antigenic and sensitizing.[(1)] These compounds have been found in several *Leishmania* species and have been shown to exhibit extensive glycosylation with phosphoglycan chains. [(2)] A major membrane surface glycoprotein, referred to as p63, has been found in *L. donovani, L. major, L. tropica*, *L. mexicana*, and *L. braziliensis*. In the native state, p63 is identified at 58 kDa, but in a reduced form it migrates to 63-65 kDa. Soteriadou et al. [(3)] postulated that the 58 kDa and 63 kDa components are the glycoprotein identified as p63. Based on the work of Khabiri et al. [(4)], it is possible that the 58 kDa material is a major contributor to the cellular hypersensitivity response to *Leishmania*. However, other compounds might also be involved as evidenced by a study which demonstrated the importance of a 30 kDa compound in sensitizing mice to *L. amazonensis* [(5)]. It is possible that p63 or the 58 kDa component of the *Leishmania* lysate might represent a protein(s) or protein-conjugate(s) which, by skin test, could detect sensitization from infection with *Leishmania*. However, it is reasonable to assume that other components of the lysate might also be important, such as the 30 kDa component [(5).] Guinea pigs studies conducted at Allermed demonstrated a high degree of cross reactivity of LtSTA in animals sensitized to L. *major* and *L. infantum*, indicating that *L. tropica, L. major* and *L. infantum* share common antigenic properties which could be related to a component, such as p63 discussed above.

4.1.5 Comparison of protein sequences of the major components to published available genomic and EST sequences of humans, other *Leishmania* species and other cross-reacting species.

The protein sequences of the 67 kDa, 58 kDa and 30 kDa, 20 kDa and 8 kDa components have been compared with the publically available information regarding the genomic and EST sequences of other *Leishmania* species, closely related parasites and humans. In as much as the *L. tropica* genome has not been completely assembled, the genome of *L. major*, a member of the *L. tropica* complex, was used as a reference for the proteins that were sequenced in LtSTA and for a broader comparison with other genomes. As expected, when comparing two highly diverged genomes, the human genome with 3 billion bases and *L major* with 30 million bases, the two genomes were quite different. Comparison on the protein level was somewhat more informative. The protein sequences identified in LtSTA were compared with the protein sequences of the human RefSeq database to see if any identified proteins had an abnormally high similarity to human protein sequences, which might suggest the possibility of cross reactivity with host proteins. None of the proteins that were identified in LtSTA had a high similarity to known human proteins. Some of the identified proteins had conserved proteases or redox domains, but these were highly diverged from the human homologues. No protein that was found in LtSTA was more similar to known human protein sequences than to a panel of highly diverged species, including Thale Cress (*Arabidopsis thaliana*), the nematode (*Caenorhabditis elegans*), pepper (*Capsicum annuum*), rice (*Oryza sativa*) and yeast (*Schizosaccharomyces japonicas*). In addition, none of the LtSTA proteins that were identified had a significantly greater similarity to human proteins than the putative dominant products in the PPD-S2 Tuberculin FDA standard. Regarding other *Leishmania* species and closely related species, the proteins identified in LtSTA at 20 kDa, 30 kDa and 56-58 kDa have a high degree of similarity to proteins of *L. braziliensis, L. infantum, L. mexicana* and to a lesser degree in *Trypanosoma cruzi* and *T. brucei*.

4.1.6 Validations Performed During Reporting Period

Validation Protocol VR2028, '*Validation of Sterile Filtration of Leishmania Skin Test Antigen*" Three separate batches were manufactured, tested for phenol, pH, sodium chloride, glycerin, phosphate, protein, potency, SDS-PAGE and ELISA before and after filtration. All testing passed the acceptance criteria and a report was written and approved.

Validation Protocol VP 2029 (VP2013 PQ), '*Validation of Cell Lysis using a 110Y Microfluidizer'* Three separate validation runs were performed to validate the Microfluidization process. Validation Report VR 2013 was written and approved.

Validation Protocol VP 2030, '*Validation Protocol for a Relative Potency Test method to evaluate the Potency of Leishmania tropica Skin test Antigen (LtSTA) with Respect to a LtSTA Internal Reference Standard (LtLRS)*' was written, the validation work executed and the data

analyzed by the statistician. The validation report VR 2030 was written by the statistician, reviewed and approved by Allermed.

Validation Protocol VP 2033, '*Ninhydrin Protein Assay'*
SOP 916-000 for the Ninhydrin procedure has been revised to incorporate improvements based on validation results. Validation Report VR 2033 was written and approved.

Validation Protocol VP2035, '*Validation of Mixing Times of Leishmania tropica Skin test Antigen Final Drug Product'* Evaluated mixing times of product formulated at both the ½ strength step as well as final formation of a *Leishmania tropica* Skin Test Antigen (LtSTA). The work was performed and the samples analyzed. The validation report VR 2035 was written and approved.

Validation Protocol VP 2036, *'Validation of the Orthophosphate Assay, Ascorbic Acid Method'*
The validation report VR 2036 was written and approved.

4.1.7 Documents Revised During Reporting Period

Document No.	Title	Rev. #
301-600	Environmental monitoring of the Leishmania Classified Areas	05
301-600F1	EM of Viable Air Particles in the Leishmania Facility	02
301-600F3	EM of Viable Surface particles in the Leishmania Facility	02
301-600F4	EM Record for Production Activities in the Leishmania Facility	02
401-003	Washing Containers and Utensils in the Bosch Dishwasher #G0032	00
413-100	Operation of the Centrifuges in the Leishmania Facility	00
413-100F1	Centrifuge Data Form	00
644-101	Bubble Point Testing in the Leishmania	00
644-101F1	Summary of Bubble Point Tests in the Leishmania Facility	00
660-008	Decontamination procedures in the Leishmania Facility	01
661-601	Batch Production Record for LtSTA Internal Reference Standard (LRS)	01
661-605	Batch Production Record for the Production of a Leishmania tropica Skin Test Antigen	01
661-605F1	Batch Production Record for the Production of a *Leishmania tropica* Skin Test Antigen	01
910-106	Leishmania Skin Test Antigen Relative Potency Test	01
910-106F1	Leishmania Skin Test Antigen Relative Potency Test Form	01
918-005	Nonviability Testing of Leishmania Parasite Derived Material	00
918-005F1	Nonviability Test Result Form	00
937-101	Bioburden Testing for Leishmania Material	00
937-101F1	Leishmania Material Bioburden Data Sheet	00
938-101	Identifying Contamination in Leishmania Cultures	00
938-101F1	Microbial Contamination in Cultures Result Form	00
944-102	Leishmania Identity Test by ELISA	01
944-102F1	Leishmania Identity Test Result Form	01

SECTION 5. CONCLUSIONS

The work performed during this reporting period was directed primarily to the chemistry, manufacture and control of LtSTA, including the characterization of major and minor proteins in the drug substance and drug product. Lot consistency and the validation of key steps in the manufacturing process were also addressed. Information concerning these issues was requested by the FDA in part because of the sensitizing capacity of the LtSTA skin test as demonstrated in Allermed's phase IIb clinical trial that was reported in the Annual Report for the 2009-2010 reporting period. In addition, FDA suggested that further characterization of the LtSTA was appropriate before another phase II or phase III clinical trial is initiated. Allermed believes that

the data that have been submitted to FDA provide this information and intends to ask that a Type B meeting be held with the FDA to discuss the present status of LtSTA development, as well as future steps relating to the design of a clinical trial that will support the use of LtSTA as a diagnostic test for cutaneous leishmaniasis.

SECTION 6. REFERENCES

1. Jose et al. Evaluation of the sensitizing power of the reaction of Monetnegro. J. Brazilian Soc. Trop. Med. 2001.

2. Klein, et al. Proteophosphoglycans of *Leishmania mexicana*. Biochemical Society, 1999.

3. Soteriadou, et al. Identification of monomeric and oligomeric forms of a major *Leishmania infantum* antigen using monoclonal antibodies. Infection and Immunity, 1988.

4. Khabiri, et al. *Leishmania major*: Common antigen responsible for induction of delayed-type hypersensitivity response in guinea pigs. Parasitol . Res., 2007.

5. Beyrodt, et al. Characterization of an antigen from *Leishmania amoazonensis* amastigotes able to elicit protective responses in a murine model. Infection and Immunity, 1997.

SECTION 7. APPENDICES

1. FDA letter dated May 7, 2010 with Allermed's reply.

2. Annual Report for BB-IND 11822.

3. FDA letter dated March 17, 2011 with Allermed's reply and 4 attachments.

4. Quarterly Reports.

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Public Health Service
Food and Drug Administration
1401 Rockville Pike
Rockville, MD 20852-1448

CENTER FOR BIOLOGICS EVALUATION AND RESEARCH
OFFICE OF VACCINES RESEARCH AND REVIEW
DIVISION OF VACCINES AND RELATED PRODUCTS APPLICATIONS

TODAY'S DATE: May 7, 2010 **PAGES:** 4

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THROUGH: **Elizabeth M. Sutkowski, Ph.D.**
Chief, Regulatory Review Branch 3

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Scientific Reviewer
FAX: (301) 827-3532 TEL: (301) 827-3070 (Voice)

SUBJECT: Review comments regarding the March 4, 2010 and March 8, 2010 amendments to ***BB-IND 11822.***

MESSAGE:

Dear Dr. Nielsen;

We have reviewed the information provided in your March 4, 2010 and March 8, 2010 submissions to your **Investigational New Drug Application (IND)** for "*Leishmania tropica* Soluble Skin Test Antigen". We have the following comments, questions, and requests for additional information:

The following comments are regarding the CMC section of the IND:

At the present time, we do not concur with your proposal to consider advancing product and clinical development to the phase 3 trial stage. We note that your clinical trials to date have enrolled small numbers of subjects; have not yet demonstrated a positive response value that achieves reliable sensitivity and specificity; and have yielded inconsistent results with regard to dose selection. These inconsistencies may be related to inconsistencies between lots of product, which have not been well characterized. Prior to proceeding to larger scale trials, we strongly recommend that you perform substantial additional product testing and characterization, with a particular view towards achieving a well-controlled and validated production process that yields a consistent product. Specifically:

1. Please perform further characterization of both the master and working cell banks. In particular, please perform appropriate testing for the presence of double stranded *Leishmania* RNA viruses, as well as other typical viral adventitious agents (which may be introduced by the use of fetal bovine serum). We refer you to the guidance for industry entitled, "*Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications*" (2010).

2. Please perform endotoxin testing of the bulk and final product, and establish appropriate specifications.

3. Please clarify whether you consider the current 1.3 ml vials to be single dose or multi-dose vials, and further justify the inclusion of phenol in the final product.

4. For all product testing, please determine estimates of error inherent to the assays themselves as well as the range of values determined for all lots of product tested to date, and revise all corresponding specifications to establish both upper and lower limits for acceptance.

5. For all product testing, please establish appropriate long term external reference standards.

6. For guinea pig potency testing, please provide data directly supporting your choice of 5 mm induration or greater as being considered positive, or revise as indicated by available data.

7. Please provide stability data clearly supporting a proposed time period for storage of bulk product before final formulation, in addition to stability data supporting the storage period of final formulated product. Further, we strongly recommend that you conduct stability testing using stressed conditions. Please comment.

8. Please conduct *bone fide* physicochemical product characterization, e.g.:

a. Determine the identities of major and minor protein components of the bulk and final products.

b. Determine the relative quantities of the major and minor protein components of the bulk and final products.

c. Establish specifications for the relative quantities of the major and minor protein components of the final product.

d. Compare the sequences of the major and minor components to human genomes, other *Leishmania* species, and other potentially cross-reacting species, and discuss the potential significance of any substantial sequence similarities/homologies you identify.

9. Please discuss the status of your efforts to validate your production process. Of note, any revisions to the production process should be implemented prior to final process validation. In particular, we strongly recommend that any changes be implemented prior to proceeding to Phase 3 trials. Please comment.

The following comments are regarding the statistical section of the IND:

10. For the future Phase 3 trial, a general statistical analysis plan (SAP) describing the study objectives, hypotheses (expressed formally with statistical notations and explanations), primary endpoints, design specifications, criteria for declaring study success and statistical analysis methods, should be submitted for CBER review. Note that the SAP is not considered final until it is concurred upon by CBER. We recommend that you not initiate a phase III trials without submitting the SAP, and that it be finalized early on during the enrollment period. This is to assure that the study design and planned analyses are adequate to meet the stated objectives. Accordingly, failure to submit the SAP until after the study has been completed and just prior to data lockdown will potentially be problematic.

The following comments are regarding the clinical section of the IND:

We have reviewed your submission containing the final study report (FSR) for study LtSTA-08, revision 03 and 03A, as well as the previously submitted Phase 1 and Phase 2 FSRs and have the following general comments:

11. These small studies are more appropriately considered Phase 1 proof of concept studies rather than Phase 2 studies. The data submitted thus far indicate insufficient characterization of the sensitivity, specificity or sensitizing properties of

the LtSTA test article. This may be related in part to insufficient characterization and inconsistent manufacturing of your product. Please comment.

12. Please clearly state your proposed indication for LtSTA including a description of how this product would be used to manage patients in a clinical setting. This information is essential for the evaluation of the risks and benefits of your product, and to guide clinical development. Please note that your proposed indication should be for "the treatment, prevention, mitigation, cure or diagnosis of a recognized disease or condition, or of a manifestation of a recognized disease or condition..." [21 CFR 201.57(c)(2)], and that a label must be supported by "substantial evidence of effectiveness" [21 CFR 201.57 (c)(2)(v)].

13. Once the broader issues of the proposed indication and the manufacture of your product are addressed, we will be better able to assist you with a clinical development plan. Please acknowledge.

If you have any questions about this communication, please contact Dr. Joseph J. Temenak at the above telephone number.

Items addressed in the May 7, 2010 letter from FDA RE: *Leishmania* Skin Test Antigen, IND 11822

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CMC

1. *Please perform further characterization of both the master and working cell banks. In particular, please perform appropriate testing for the presence of double stranded Leishmania RNA viruses, as well as other typical viral adventitious agents (which may be introduced by the use of fetal bovine serum). We refer you to the guidance for industry entitled, "Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications" (2010).*

We have reviewed the guidance document cited above concerning the characterization and qualification of cell banks. It is our understanding that the work that has been completed on the master and working cell banks of *Leishmania tropica* (WR1063) addresses the recommendations of the guidance document.

The tests that were performed on isolate WR1063 and the testing facilities are shown below. Please refer to Attachments A-F for the test reports. You specifically mention *Leishmania* RNA viruses. This work was done by the Southwest Foundation for Biomedical Research on behalf of the United States Army (WRAIR Pilot Bioproduction Facility, Silver Springs, MD) prior to sending a seed culture of the parasite to Allermed. We note that the recommendations listed in the guidance document are directed towards cell substrates and other biological materials that are used in the production of viral vaccines. It is not clear to us how some of the recommendations mentioned in the guidance document apply to a skin test product prepared from *L. tropica*. The table below lists the tests that Allermed has performed on isolates WR1063 and the cell banks that were made from the organism.

Description
Assays for Leishmania virus RNA Performed on *Leishmania tropica* Seed Material (WR1063), Cell Banks, Raw Material & FBS using TaqMan RT-PCR also Nuclear Hybridization
Speciation of Leishmania Parasites by Isoenzyme Analysis
Assays for Mycoplasma Detection Performed on *Leishmania tropica* Seed Material (WR1063), Cell Banks, Raw Material & FBS: "Points to Consider" Using Indirect Staining and Direct Microbiological Assay
Test for the Presence of Non Agar Cultivable Mycoplasma using Vero Cell Culture Assay
Assays Performed on *Leishmania tropica* Cells Beyond the Production Age Limit: Test for the Presence of Agar Cultivable Mycoplasma
Assay for the Presence of Bovine Viruses in *Leishmania tropica* Parasites

2. *Please perform endotoxin testing of the bulk and final product, and establish appropriate specifications.*

Section 21 CFR 610.13 states that endotoxin testing is not required for diagnostic products. Since the indicated use of LtSTA is to identify persons with Type IV hypersensitivity resulting from infection with *L. major*, it should be considered a diagnostic product. Please clarify FDA's position on endotoxin testing for these types of biologicals.

3. *Please clarify whether you consider the current 1.3ml vials to be single dose or multi-dose vials, and further justify the inclusion of phenol in the final product.*

LtSTA is filled in a 2.0 mL multi-dose vial. The fill volume is 1.3 mL which provides enough material for ten 0.1 mL doses, including a suitable overfill. The vial contains phenol which is an accepted preservative for containers intended for multi-dose use.

4. *For all product testing, please determine estimates of error inherent to the assays themselves as well as the range of values determined for all lots of product tested to date, and revise all corresponding specifications to establish both upper and lower limits for acceptance.*

The range of values for the components of LtSTA is based on the results of product testing of seventeen lots (Table 4.1). The lot size and the protein concentration of the lot varied depending upon the intended use of the lot. Initially, the product was made at a protein concentration of 0.6 mg/mL based on information provided in a previously published study which showed that *Leishmania* skin test antigen, in this dose range, was safe and effective in detecting delayed-type hypersensitivity to *Leishmania.* [(1)] Three lots of LtSTA manufactured at a protein concentration of 0.6 mg/mL were enrolled in a stability protocol that has been followed for six (6) years. Four additional lots were made at 0.6 mg/mL for validation purposes. Two lots of the product were made at a protein concentration of 1.2 mg/mL to provide material to conduct phase 1 and phase 2 clinical trials. In both trials, the product was diluted at the clinical site to the concentration specified in the study protocol. One lot of product was manufactured at a protein concentration of 0.3 mg/mL for use in a phase 2B study, and two lots were manufactured at a protein concentration of 0.5 mg/mL for use in the same phase 2B investigation.

The inherent error of each assay that is used in measuring the concentration of excipients and other attributes of LtSTA is reflected in the value obtained for each assay. The sensitivity and specificity of the assay and the variability associated with the performance of the assay are reflected in the final result of the test procedure. With respect to LtSTA, all of these variances are recognized in data collected from seventeen lots of the product. The high and low values (range) for the excipients are reported in Table 4.0 below.

Table 4.0 Range of Test Results for Excipients and pH for Seventeen Lots of LtSTA

Test	*Low Value*	*High Value*
Phenol	0.40 %	0.43 %
Glycerin	0.87 %	0.94 %
Sodium Chloride	0.87 %	0.92 %
pH	6.8	6.9
BSA	21.0 ng/mL	106.0 ng/mL
Phosphate	2.16 mg/mL	2.76 mg/mL

The data shown in Table 4.0 were obtained from the results of assays performed on seventeen lots of LtSTA shown in Table 4.1.

Table 4.1 Product Testing Results for Seventeen Lots of LtSTA

Lot #	Target Protein (mg/mL)	Phenol (0.40%) [0.34-0.46	Glycerin (1.0%) [0.85-1.10]	NaCl (0.85%) [0.80-0.95]	pH (7.0) [6.5-7.5]	Measured Protein (mg/mL)	Safety	Potency (mm)	BSA [<500 ng/mL]	Phosphate [2.07-2.67 mg/mL]	Final Sterility Test
XLtSTA001	0.69	0.41	0.87	0.65*	6.9	0.65	Pass	15	19.6	2.76	Pass
XLtSTA002	0.60	0.45	0.90	0.88	6.9	0.60	Pass	14	30.2	2.42	Pass
XLtSTA003	0.56	0.41	0.94	0.87	6.9	0.61	Pass	12	18.3	2.32	Pass
XLtSTA006A	0.59	0.42	0.92	0.91	6.8	0.59	Pass	11	38.7	2.16	Pass
XLtSTA006B	1.20	0.43	0.90	0.91	6.8	1.12	Pass	ND*	69.85	2.25	ND
XLtSTA006C	1.20	0.43	0.92	0.91	6.8	1.0	Pass	14	80.3	2.27	Pass
XLtSTA007	0.59	0.42	0.92	0.87	6.8	0.64	Pass	13	72	2.34	Pass
XLtSTA008	0.70	0.40	0.92	0.89	6.8	0.70	Pass	12	187	2.31	Pass
XLtSTA009	0.60	0.43	0.90	0.87	6.8	0.62	Pass	12	29	2.59	Pass
XLtSTA010	0.60	0.42	0.89	0.89	6.8	0.58	Pass	12	106	2.57	Pass
XLtSTA012	1.20	0.43	0.91	0.88	6.8	1.15	Pass	12	76	2.29	Pass
XLtSTA013	1.00	0.41	0.91	0.88	6.8	1.16	Pass	11	37	2.48	Pass
XLtSTA014	1.20	0.41	0.91	0.89	6.8	1.25	Pass	12	54	2.45	Pass
XLtSTA016	0.50	0.42	0.87	0.88	6.9	0.51	Pass	11	23	2.61	Pass
XLtSTA017	0.50	0.42	0.89	0.88	6.9	0.53	Pass	11	21	2.54	Pass
XLtSTA018	0.30	0.42	0.90	0.89	6.9	0.31	Pass	10	44	2.39	Pass
XLtSTA019	0.14	0.42	0.90	0.89	6.9	0.14	Pass	8	30.6	2.26	Pass

*Incomplete processing of this lot

The value for sodium chloride in Table 4.1 for lot XLtSTA001 (0.65%) was an error. This value was omitted from Table 4.0. Potency was not included in Table 4.0, since the lots were at different protein concentrations. In Table 4.1, values entered in the potency column refer to the mm induration response to the concentration shown in the table. However, in the manufacture of future lots, the potency of the product will be based on a standardized relative potency assay which involves induration measurements from four 1:3 log dilutions of the product. The same four dilutions of the internal standard (LRS) are prepared and the induration responses to the two dilutions series are compared in a parallel-line bioassay. The mm

induration values shown in Table 4.1 are for the undiluted product; these values provide some indication of potency, but differences in product potency are more apparent with titration dilutions as used in the relative potency method (Attachment G).

Table 4.2 Values for Excipients & pH for 17 Lots of LtSTA Showing the Average, Standard Deviation & the 3 sigma for each Component

		phenol		Glycerin		NaCl		pH		Phosphate	
	Lot #										
1	XLtSTA001	0.41		0.87		0.65*		6.9		2.76	
2	XLtSTA002	0.45		0.90		0.88		6.9		2.42	
3	XLtSTA003	0.41		0.94		0.87		6.9		2.32	
4	XLtSTA006A	0.42		0.92		0.91		6.8		2.16	
5	XLtSTA006B	0.43		0.90		0.91		6.8		2.25	
6	XLtSTA006C	0.43		0.92		0.91		6.8		2.27	
7	XLtSTA007	0.42		0.92		0.87		6.8		2.34	
8	XLtSTA008	0.40		0.92		0.89		6.8		2.31	
9	XLtSTA009	0.43		0.90		0.87		6.8		2.59	
10	XLtSTA010	0.42		0.89		0.89		6.8		2.57	
11	XLtSTA012	0.43		0.91		0.88		6.8		2.29	
12	XLtSTA013	0.41		0.91		0.88		6.8		2.48	
13	XLtSTA014	0.41		0.91		0.89		6.8		2.45	
14	XLtSTA016	0.42		0.87		0.88		6.9		2.61	
15	XLtSTA017	0.42		0.89		0.88		6.9		2.54	
16	XLtSTA018	0.42		0.90		0.89		6.9		2.39	
17	XLtSTA019	0.42		0.90		0.89		6.9		2.26	
		LL	UL	LL	UL	LL	UL	LL	UL	LL	UL
	avg	0.421		0.904		0.873		6.841		2.412	
	std dev	0.011		0.018		0.059		0.051		0.160	
	3sigma	**0.39**	**0.45**	**0.85**	**0.96**	**0.70**	**1.05**	**6.69**	**6.99**	**1.93**	**2.89**

* excluded from analysis

Bovine Serum Albumin (BSA) varied from 18.3 ng/mL to 187 ng/mL in the seventeen manufactured lots. However we have adopted a limit of <500 ng/mL from the July 12, 1993 Memorandum regarding *Points to Consider in the Characterization of Cell lines Used to Produce Biologicals*, page 13 which states that the amount of residual animal serum in the final product should not exceed 1:1,000,000.

We have used the values reported in Table 4.1 and Table 4.2 to establish limits for the excipients and the pH of the final product. Lot XLtSTA006B was vialed, stored at 1-5 °C without potency or sterility testing and was eventually discarded. However, the lot was included in Table 4.2 since the data for other attributes were available. The limits shown in Table 4.2 are based on three +/- standard deviations of the mean. Manufacturing SOPs have been revised to include the limits shown at the bottom of Table 4.2 for phenol, glycerol, NaCl, pH and phosphate.

5. *For all product testing, please establish appropriate long term external reference standards.*

There are no external standards for this product. Allermed has manufactured a *Leishmania tropica* reference standard (LRS) at a protein concentration of 0.5mg/mL. The reference standard has been dispensed into 2 mL vials which are stored at -190 °C in two different cryotanks containing liquid nitrogen. The reference standard will be used in testing the identity and potency of the product. A sufficient number of vials have been made to last approximately ten years.

Allermed has obtained a sufficient volume of anti-*Leishmania tropica* rabbit serum for use in the ELISA identity test to last approximately ten years. This antiserum is stored at -20 °C in two different freezers. New lots of antisera will be compared with the existing lot when the supply of the current reference is low.

6. *For guinea pig potency testing, please provide data directly supporting your choice of 5 mm induration or greater as being considered positive, or revise as indicated by available data.*

Induration equal to or greater than 5 mm is considered a positive DTH response in humans. Recognizing 5 mm induration as a positive response is based on the results of DTH skin testing over decades of use in the medical and scientific communities. In guinea pigs, 5 mm of induration is a minimal reaction, but large enough to distinguish the response from that of placebo. In sensitizing guinea pigs for use in the potency assay of LtSTA, an 8 mm induration response to the reference standard is considered adequate for enrollment in the assay. We are currently following a protocol that involves a parallel-line bioassay in which dilutions of a manufactured lot are tested in parallel with the reference standard (LRS). This potency assay has been validated in the development of another skin test antigen at Allermed, and it is currently being followed for this product. A copy of the method is enclosed as Attachment G.

7. *Please provide stability data clearly supporting a proposed time period for storage of bulk product before final formulation, in addition to stability data supporting the storage period of final formulated product. Further, we strongly recommend that you conduct stability testing using stressed conditions. Please comment.*

The bulk drug product is formulated from the sterile promastigote lysate (bulk drug substance). Following formulation, it is stored at 1-5 °C until protein and phenol assays are performed. If the protein and phenol levels are within specification, the bulk drug substance is aseptically filtered and held for at least 14 days to complete the bulk sterility test. During this time, the protein concentration and the potency of the bulk drug product are measured. Stability testing of the bulk drug substance is not done, since the bulk drug product is not retained, but is filled in final containers once the bulk sterility test and the protein and potency assays have been completed. The time permitted to process bulk drug product following formulation, aseptic filtration and quality assurance testing is six (6) weeks. During this time the bulk product is

stored at 1-5 °C. Any change in the stability of the product at the bulk stage of manufactured would be reflected in the results of stability tests on the product in final containers. Bulk drug product has been held at 1-5 °C for as long as 72 days before being filled into final containers. In all instances, the drug product manufactured from these bulk materials met release specifications.

Data for three lots of LtSTA manufactured at a protein concentration of 0.6 mg/mL are available to support stability for 5 years when the product is stored at 1-5 °C. During this validation study, the product was stored under stress conditions at elevated temperatures of 23-27 °C and 35 °C. We are currently accumulating stability data on final product manufactured at a protein concentration of 0.5 mg/mL. Based on our present understanding of the stability of this product, we do not believe that product manufactured at a protein concentration of 0.5 mg/mL will differ significantly from product manufactured at a protein concentration of 0.6 mg/mL. However, we intend to obtain real-time data for product containing 0.5 mg/mL of protein that will support an expiration date of at least 3 years when the product is stored at 2-8 °C.

Accelerated or stress testing is included in the stability testing of this product. Protocol 949-025 (Attachment H) describes the testing that will be done on the product in final containers. Three elevated temperatures will be used to challenge the stability of the product (2-8 °C, 23-27 °C and 35-40 °C). Containers are stored at these temperatures in the upright and inverted positions.

8. Please conduct bona fide physicochemical product characterization, e.g.:

a. Determine the identities of major and minor protein components of the bulk and final products.

LtSTA is a sterile solution made from the crude lysate of the promastigotes of *L. tropica*. The product is capable of detecting delayed-type (IV) hypersensitivity following cutaneous infection with *L. major*. We have not attempted to fractionate the extract, since our objective is to manufacture a product that is representative of the total antigenicity of the organism. The skin test antigens of *Leishmania* that have been used by medical and scientific investigators throughout the world are either whole promastigotes or soluble lysates of promastigotes. These preparations have been found to be safe and efficacious as skin test antigens (2-15). We believe that LtSTA also can be used successfully as a whole lysate. Data from the phase 2 clinical trial conducted by Allermed show that LtSTA has acceptable sensitivity and specificity in sensitized and non-sensitized populations, respectively.

SDS-PAGE is included in the manufacturing process of LtSTA as a first-step identity test to confirm that the product being manufactured has a protein banding profile that is similar to the reference standard (LRS). The procedure is not intended to identify major or minor proteins in the lysate. Over the course of developing LtSTA, we have observed a banding pattern that is

similar from lot to lot. Initially, we attempted to identify specific bands in the crude lysate and in the finished product which could be used as identity markers. However, after reviewing all of the data from the work performed at Allermed, it is our opinion that the protein profile obtained with SDS-PAGE can provide useful information if the entire banding pattern is used, rather than focusing on specific bands. An example of a characteristic profile of LtSTA is shown in Figure 1 in which three groups of bands are present. Group 1 is found between 4 KDa and 16 KDa. Group 2 is found between 20 KDa and 36 KDa and Group 3 is found between 50 KDa and 98 KDa. Each group consists of several to many bands with varying degrees of staining intensity. When considered collectively, the three groups of protein bands appear to be somewhat consistent from lot to lot in the crude lysate. It is our opinion that SDS-PAGE should be used in the manufacture of LtSTA in the same manner that isoelectric focusing is used in the manufacture of standardized allergenic extracts. In both instances, the procedures are fingerprint techniques that are useful in the identification of the product, but are not the primary identity tests.

We have subjected the whole-cell lysate to a temperature of 90 °C for 60 minutes in an attempt to alter the banding profile, and to see if changes in the profile could be correlated with changes in the potency of the product. However, these experiments were unsuccessful in that a correlation between specific SDS-PAGE bands and potency was not observed. We also compared the banding profiles of production lots of LtSTA manufactured approximately eight years ago with newly manufactured lots. Some loss of band intensity occurred in older lots, but the loss of a specific band, or of the staining intensity of a specific band did not correspond with a loss in potency. SDS-PAGE is not used as a release criterion in the manufacture of LtSTA, nor is it an indicator of stability.

Examples of crude preparations of biological materials that have been licensed by the FDA as skin test antigens include allergenic extracts of house dust mite, cat hair, ragweed pollen and grass pollens. Major allergens, such as Der p 1 and Der p 2 in the extract of house dust mite *Dermatophagoides* have been identified. However, the FDA approved skin test product of *Dermatophagoides* is the crude extract of the mite, standardized by ELISA using human antiserum obtained from mite allergic individuals. Amb a 1 is considered to be the major allergen in short ragweed extract. However, the FDA approved product for the diagnosis of ragweed allergy is not a preparation of Amb a l, but rather, it is the crude short ragweed extract containing the entire complement of short ragweed allergens.

Khabiri, Bagheri and Assmar (16) found that a 56 KDa band in the lysate of *L. major* promastigotes appeared to be associated with a positive DTH response in sensitized guinea pigs, but to a lesser degree than the whole-cell lysate. These authors also site studies that show the immunogenicity of various fractions. However, we have been unable to confirm a direct relationship between the protein bands in the 56 KDa region of LtSTA with the potency of the product. We have discussed the characterization of the major and minor proteins of LtSTA with several firms that provide this service. It is questionable that this information would enhance the safety or efficacy of the product. The isolation and characterization of lysate fractions that are

immunologically active in the DTH response would add a significant barrier to completing the development of LtSTA.

b. Determine the relative quantities of the major and minor protein components of the bulk and final products.

The crude lysate of *L. tropica* promastigotes has been shown to be safe and effective in phase 1 and phase 2 controlled studies. Moreover, the data from these studies demonstrate that LtSTA has the sensitivity and specificity required for clinical use. We do not have clinical data on fractions of the lysate that might include immunologically active proteins. As stated in 8.a isolating fractions of the promastigotes and using such fractions in a diagnostic capacity runs the risk of missing infected persons in which their immune system fails to recognize the fraction(s). In nature, sensitization occurs from contact with the whole organism. For this reason, we believe that it is advisable to use the whole lysate containing the entire antigenic complement of the parasite.

c. Establish specifications for the relative quantities of the major and minor protein components of the final product.

Please see our response to 8.a and 8.b above.

d. Compare the sequences of the major and minor components to human genomes, other Leishmania species, and other potentially cross-reacting species, and discuss the potential significance of any substantial sequence similarities/homologies you identify.

We know from Allermed's studies in guinea pigs and humans that cross reactions occur. This was demonstrated with LtSTA in guinea pigs sensitized to *L.major* and *L.infantum* and in humans sensitized with *L. major*. In order to mitigate the risk of misdiagnosis with cross-reacting agents, Allermed is recommending that the LtSTA skin test is only used as a first step diagnostic. To confirm infection by *Leishmania*, the organism must be isolated and cultured using standard laboratory methods.

Complete genomic sequencing of *L. tropica* is not currently available. The genomes of *L. major, L.infantum* and *L.braziliensis* have been sequenced resulting in more than 8300 protein-coding and 900 RNA genes.[17] Assuming that the major and minor components of the crude lysate of *L. tropica* were identified, the additional burden of identifying the relevant genomic sequences in order to address this comment (8.d) would still exist. Genome characterization efforts are not trivial and usually involve cooperative studies between various investigational groups or institutions, such as the Leishmania Genome Network (LGN) which includes the Seattle Biomedical Research Institute, the Welcome Trust Sanger Institute, the Vancouver BC Genome Centre and other research centers from various locations in various countries throughout

the world.[17] In our opinion, providing the information required to fully address this comment goes beyond reasonable expectations for the development and licensure of a skin test antigen, considering the route of administration, dose and frequency of use.

9. *Please discuss the status of your efforts to validate your production process. Of note, any revisions to the production process should be implemented prior to final process validation. In particular, we strongly recommend that any changes be implemented prior to proceeding to Phase 3 trials. Please comment.*

The LtSTA manufacturing process was developed from a series of carefully controlled studies beginning with the selection of the cultivation medium, passage of the organism from the working cell bank to production cultures, growth of parasite in Celstirs, harvesting and washing promastigotes, microfluidization of promastigotes, heating the lysate at 60 °C, and formulation of the drug product. Studies relating to these manufacturing steps are reported in the following documents on file at Allermed:

Document #	***Description***
ER006	Media Comparison for Growing *Leishmania tropica* Parasites
ER003	Growth Phase Determination of *Leishmania tropica*
ER009	Optimizing Growth Conditions of Parent Cultures for Inoculation of Production Cultures
ER008	Optimization of Parasite Growth in Spinning Cultures
ER018	Optimization of Atmospheric Growth Conditions for *Leishmania tropica*: Filtered Air
ER005	Parasite Harvest and Washing: Tangential Flow Filtration vs. Centrifugation
ER004	Parasite Sensitivity to Heat
ER012	LtSTA Formulation Study

The following validations of the facilities, equipment and processes relating to the manufacture and testing of LtSTA have been completed, or in the process of being completed. Validation protocols and reports are on file at Allermed.

Validation#	***Description of Equipment & Facility Validations***
2000	Validation Master Plan Leishmania Facility Validation
2001	VWR -80 Freezer qualification, Equipment # G0016
2002	Thermolyne Locator 8 Plus (LN2) Vessel Qualification, Equipment # G0002
2003	Kaye Instrument Validator 2000 Qualification, G0058/G0059
2004	The Leishmania Cleanrooms Validation
2005	GETINGE GMP Autoclave Validation, Equipment # G0042
2006	LMS PASSPORT Autoclave Validation, Equipment # G0043
2007	Forma Environmental Chamber (Incubator) Qualification, Equipment # G0037
2008	Walk-In Cooler Qualification, Equipment # G0062

Validation#	*Description of Equipment & Facility Validations*
2009	Smartspec 3000 Spectrophotometer Qualification, Equipment # G0041
2010	SPECTRAmax 340PC Plate Reader Qualification, Equipment # G0052
2011	Eppendorf 5810R Centrifuge Validation, Equipment # G0012
2012	Jouan KR422 Centrifuge Validation, Equipment # G0061
2013	Microfluidics Microfluidizer Validation, Equipment # G0053
2014	Labconco Biological Safety Cabinet # G0015
2015	Labconco Biological Safety Cabinet # G0063
2016	Honeywell Multi-trend plus V5 Monitor # G0094
2017	Leishmania Facility DI Water System Validation
2018	Fisher Hamilton SafeAire Fume Hood Equipment no. G0082
2019	Barnstead LOCATOR 8 Plus Cryobiological Storage Vessel, Equipment no. G0115
2025	Barnstead Lab-Line Imperial III General Purpose Incubator, Equipment no. G0131
2027	ThermoLabsystems Well Wash 4 Mk 2 Microplate Washer, Equipment no. G0132

Validation#	*Description of Process and Test Method Validations*
2021	Sterility Test Validation of Leishmania tropica Product and Material: Bacteriostasis and Fungistasis
2022	Preservative Effectiveness Testing for LtSTA
2023	Hold Time Media
2024	Validation Protocol for Sodium Chloride Analysis: Titrimetric Assay
2026	Holding Time Validation for Leishmania Product Solutions
2028	Validation of Sterile Filtration of Leishmania Skin Test Antigen
2029	Validation of Cell Lysis Using a 110Y Microfluidizer
2030	Validation of Relative Potency Test Method to Evaluate the Potency of LtSTA with Respect to a LtSTA Internal Reference Standard (LRS)
2033	Validation of Ninhydrin Protein Assay for use with LtSTA
2034	Validation of the Leishmania tropica Skin Test Antigen Identity Test (ELISA)
2035	Validation of Mixing Times of Leishmania tropica Skin Test Antigen Final Drug Product

The assays that are used in measuring the level of salts and preservative in LtSTA are routinely used at Allermed in the testing of currently licensed products. The data obtained from the use of these procedures in the testing of LtSTA have been consistent from lot to lot, which demonstrates that the procedures yield uniform results when the parameters of the test remain unchanged as described in the SOPs for each assay.

The general method of manufacture of the drug substance and drug product has been consistent throughout the development process. Aeration was added to Celstir cultures to increase the yield of the parasite and the procedure was validated, but no other procedural changes have been introduced in the manufacturing process of the promastigotes lysate. The concentration of the drug product has varied, depending upon the intended use of the skin test antigen. However, no other changes have been introduced.

To illustrate the consistency of the manufacturing process, data for two lots of LtSTA made at a protein concentration of 0.5mg/mL are shown in the Tables 9.1-9.4. Table 9.1 shows the processing data for the steps involved in the growth and harvesting of promastigotes, beginning with the reactivation of the working cell bank (WCBLt01) and progressing through the harvesting of promastigotes from Celstir cultures. Table 9.2 shows the data obtained in the processing of promastigotes (frozen at -80 °C after harvesting) including heating at 60 °C, non-viability testing and protein analysis. Table 9.3 shows data associated with the preparation of the bulk drug product and Table 9.4 includes the final container test data. The procedures followed in each of the manufacturing steps are discussed below. A diagram of the manufacturing process is shown in Figure 2.

1. Cultivation and Harvest of Promastigotes

A single vial of the *Leishmania tropica* working cell bank is withdrawn from liquid nitrogen storage. The frozen cell bank is reactivated by aseptically transferring the parasite pellet to 5 mL of Schneider's Medium with 10% Fetal Bovine Serum (FBS) contained in a T25 flask. Percent viability is determined microscopically by counting non-viable cells and recording the results in the batch record.

Approximately 24 hours after reactivation, the T25 flask is observed for promastigotes in the mid to late log growth phase. The cell concentration is determined and the volume of inoculum is calculated (must be $\geq 4 \times 10^6$) prior to being transferred to a T150 flask containing 60mL of Schneider's medium. Earlier studies used 50mL of Schneider's at this step, but the amount of medium was changed to 60 mL to increase the culture volume in the flask.

Approximately 48 hours after a T150 flask is inoculated, the culture is observed for promastigotes in mid to late log growth phase. Cell concentration is determined and inoculum volume is calculated as described above. The promastigotes are then transferred to two T300 flasks, each containing 450 mL of Schneider's Medium. Earlier studies used 500mL of Schneider's Medium, but the volume was reduced to 450 mL, due to the possibility of the culture being exposed to the cap of the flask and creating a potential contamination risk.

Approximately 48 hours after the T300 flasks are inoculated, samples are withdrawn from each flask and placed in T25 flasks to observe for promastigotes in mid to late log growth phase. If acceptable growth is observed, the contents of the flasks are combined, cell concentration determined and the inoculum volume is calculated. The inoculum volume is then placed into a 3-liter spinner flasks (Celstir®) containing 1.5 liters of Half Schneider's medium with 5% FBS. The Celstirs are fitted with a double hose-barbed apparatus with sterile vent filters. The Celstirs are placed on magnetic stir plates set at 80 rpm and medical grade air is supplied through the vent filter directly into the medium at approximately 1 bubble per second.

Approximately 72 hours after the Celstir cultures are initiated, samples are withdrawn from each Celstir culture and placed in T25 flasks to observe the stationary growth phase of development. Both cell concentration and total cell numbers are determined. The cells are

harvested and then washed with sterile saline by centrifugation. Bioburden samples are taken prior to the final centrifugation run. The pellet is stored at -80 °C until released for further manufacturing. Data for two lots of raw material that were used in the preparation of 0.5 mg/mL product are reported in Table 9.1.

Table 9.1: Cultivation and Harvest of Promastigotes (Raw Material)

Raw Material Lot Number*	XLtSTA016	XLtSTA017
Reactivation		
Seed Lot Number	WCBLt01	WCBLt01
Reactivation Date	11/28/06	07/17/07
% Viability	99	93
T25 to T150		
Inoculation Date	11/29/06	07/18/07
Growth Phase	mid-log	mid-log
Cell Concentration cells/ml	$1.08x10^8$	$9.17x10^7$
T150 to T300		
Inoculation Date	12/01/06	07/20/07
Growth Phase	late-log	mid-log
Cell Concentration cells/ml	$7.71x10^7$	$1.05x10^8$
T300 to Celstirs		
Inoculation Date	12/04/06	07/23/07
Growth Phase	mid-log	mid-log
Cell Concentration cells/ml	$5.07x10^7$	$4.89x10^7$
Harvest		
Harvest Date	12/07/06	07/26/07
Growth phase	stationary	stationary
Pre-Harvest Cell Concentration	$6.38x10^7$	$5.94x10^7$
Pre-Harvest Total Cells	$8.26x10^{11}$	$7.71x10^{11}$
Post Harvest Total Cells	$6.35x10^{11}$	$5.66x10^{11}$
Percent Recovery	77	73
Non-motile Cells	3	5
Granular Cells	3	5
Characteristic of *L. tropica*	yes	yes
Bioburden Results CFU/mL	0	0

*Raw Material lot number is designated as the genus and species followed by the date of reactivation.

All manufacturing steps described above are performed in a Class II safety cabinet by personnel dressed in sterile cleanroom garments. Cultivation of the parasite occurs in a dedicated 25 °C incubator.

2. Preparation of the Drug Substance

A frozen pellet of *L. tropica* promastigotes is removed from -80°C storage and suspended in sterile Saline with 0.0001% Tween 80 (SALT). While one technician is thawing the pellet, a second technician is setting up and preparing to microfluidize the material in a Class II safety cabinet. The flow rate is determined by running the previously sterilized microfluidizer at operating pressure for one (1) minute using the sterile SALT solution and collecting the volume

in a graduated cylinder. The processing time is calculated using flow rate and a ten (10) pass production scheme. Once the pellet is completely thawed, the sterile SALT solution is removed from the microfluidizer and replaced with the promastigotes suspension.

Microfluidization occurs for approximately 5 minutes. The lysate is collected in sterile centrifuge tubes and centrifuged for 30 minutes at 18,500xg. The supernatant is decanted into a sterile glass bottle and the pellet is discarded. The lysate is then heated in a water bath for 30 minutes at 60 °C. The material is cooled to 10 °C, poured into sterile centrifuge tubes and centrifuged at 18,500xg for 30 minutes. The supernatant is retained and the pellet discarded. The supernatant is aseptically filtered using a 0.2µm cellulose acetate filter and retained in a sterile 500mL bottle. Samples are withdrawn for non-viability testing, protein analysis and SDS-PAGE. The lysate is stored at 2-8 °C until released for further manufacturing. These procedures are summarized in Table 9.2 with data for two lots of drug substance.

Table 9.2: Processing Data for the Preparation of Promastigote Lysate

Microfluidization		
Product Lot Number*	**XLtSTA016**	**XLtSTA017**
Date Removed from -80 °C	06/19/07	08/15/07
Flow Rate mL/1 min	400	380
Processing Time min	5 min 23 sec	5 min 55
Highest Gauge Reading psi	85	85
Actual Process PSI, High	19940	19700
Actual Process PSI, Low	19660	15120
Centrifugation # 1		
Centrifuge Time	30 min 0 sec	30 min 0 sec
Actual High Temp °C	12	14
60°C Heating		
Time Held at 60°C	30 min 0 sec	30 min 0 sec
Time to Cool	5 min 24 sec	4 min 43 sec
Total Process Time	35 min 24 sec	34 min 43 sec
Centrifugation # 2		
Centrifugation Time	30 min 0 sec	30 min 0 sec
Actual High Temp °C	12	14
Filtration and Testing		
Drug Substance (mL)	275	290
Nonviability Test	pass	pass
Ninhydrin Protein Concentration mg/ml	2.6	2.03

*The Drug Substance lot number is assigned on the day of Microfluidization and is designated as the genus and species followed by STA (skin test antigen) followed by the next consecutive number from the lot number log.

3. Preparation of the Bulk Drug Product

The drug product is prepared by aseptically combining the drug substance (promastigotes lysate) with an equal volume of 2X phosphate diluent. The lysate and 2X phosphate diluent are mixed for 10 minutes using a magnetic stirring device. The protein concentration of the diluted

lysate is determined and the lysate is further diluted to the desired protein concentration (0.5 mg/mL) with 1X phosphate diluent containing10% Tween 80. The solution is again mixed for 10 minutes and resampled for protein content to ensure that the correct dilution was made. Phenol, present as the preservative in both diluting buffers, also is measured at this point in the manufacturing process.

The bulk drug product is tested for bioburden and aseptically filtered using a sterile 0.2µm cellulose acetate filter and peristaltic pump as described below. Following filtration, samples are taken for the bulk sterility test (described below), in-process protein determination and in-process potency testing. The bulk drug product is stored at 1-5 °C until further processed in final containers.

Aseptic Filtration of the Bulk Drug Product

The drug product is formulated in a 20 liter glass carboy in a Class 100 Vertical Laminar Flow (VLF) HEPA workstation located in the filtration room of the sterile processing facility. Once formulated, two operators decant the solution into a sterile stainless steel pressure vessel. A third operator removes a sterile Sartorius cellulose acetate 0.22 µm capsule filter assembly from a sterile pouch and aseptically connects it to the pressure vessel. The filter capsule is attached to a bacteriological bell which is placed over the mouth of a sterile receiving glass carboy. Transfer of the bulk drug product from the pressure vessel to the receiving carboy is accomplished with sterile filtered nitrogen gas at a pressure of 20psi. When aseptic filtration is finished, the bacteriological bell is removed and the mouth of the receiving carboy is covered with sterile aluminum foil to prevent contamination of the contents. The filter is bubble point tested and results are recorded in the batch record. A microbiologist is responsible for air, surface and personnel monitoring, as well as recording the lot numbers of all components used in the filtration process. Air quality is monitored using a SMA MicroPortable viable particle counter and a Met One laser particle counter. A production supervisor oversees the filtration process to ensure adherence to proper procedures and aseptic technique.

At the conclusion of the filtration process, TSA/Lecithin/Polysorbate-80 touch plates are used to sample the sleeves and gloves of filtration technicians. The plates are incubated for seven days at 30 – 35°C; the results are read and recorded. The QCU is responsible for the analysis of results and for determining if follow-up is required. Deviations from established procedures are documented. All deviations must be justified or the product must be discarded.

Sterility Test of Bulk Drug Product

A 20mL sample of the aseptically filtered bulk drug product is removed from the receiving carboy using a sterile pipettor and 25mL disposable pipette. This procedure is performed in a Class 100 VLF workstation by an operator who is fully gowned in sterile cleanroom garments, sleeves and gloves. A 10mL aliquot is added to 400mL of sterile

Trypticase Soy Broth Medium (TSB) and a 10mL aliquot is added to 400mL of sterile Fluid Thioglycollate Medium (FTM). The media are labeled with the sample date and lot number of the diluent and incubated at 20-25°C and 30-35°C, respectively. The gloves and sleeves of personnel performing the bulk sterility test are monitored with touch plates as discussed above in section (1). The carboy containing the sterile bulk drug product is placed inside two sterile plastic bags which are sealed to prevent contamination of the glass surface of the carboy. The bulk drug product is removed from the filtration room and stored at 1-5°C inside the sterile processing facility for 14 days until the result of the bulk sterility test is known. The 1:40 ratio of product to medium (10mL:400 mL) is a validated procedure based on dilution of the phenol preservative to a non-inhibitory concentration when challenged with the microorganism specified in the USP. Table 9.3 shows processing data for two lots of bulk drug product.

Table 9.3: Processing Data for Bulk Drug Product

Product Lot Number	XLtSTA016	XLtSTA017
Formulated	8/2/07	8/27/07
Protein (mg/mL)	0.49	0.50
Phenol (0.40% w/v)	0.43	0.43
Bulk Sterility Test	Pass	Pass

4. Preparation of Final Drug Product

The preparation of the final drug product involves dispensing the bulk drug product into final containers. This process is performed in a dedicated, limited access, classified area. A vigilant and responsive program for environmental monitoring is followed to assess the effectiveness of the cleaning and sanitization procedures. The filling, capping and assembly of final containers occur in Class 100 workstations furnished with VLF HEPA filters which are located in Class 10,000 rooms.

To ensure product sterility, operators are trained to follow aseptic processing procedures. Personnel involved in the filling operations are trained in gowning, aseptic technique, hygiene, and cleanroom behavior. Prior to entering the aseptic processing area, operators dressed in sterile clean room garments are assisted in donning sterile sleeves and gloves and assisted in entering the sterile processing room. The aseptic processing of LtSTA involves the following procedures:

Staging for sterile filling begins with the removal of the carboy containing the sterile bulk drug product from 1-5 °C storage and transferring it to the entrance of the filling room. The outer plastic bag is removed and the bulk product is then taken into the filling room where the second plastic bag is removed under a Class 100 VLF HEPA hood. Sterile tubing connected to the dispensing pump is then aseptically placed in the bulk product. At this point in the process, the bulk drug product is ready to be dispensed into final containers.

At the start of the filling operation, temperature and relative humidity readings from a Met-One particle counter and differential pressure from a magnehelic gauge are recorded in the Batch Production & Control Record (BPCR).

During the fill, positive air pressure in the Filling Room is continuously monitored by an electronic Honeywell Multitrend Recorder. The temperature (range 64-76 °F) and humidity (range ≤65%) also are read and recorded in the BPCR.

Environmental monitoring of the air for viable particles is achieved using a SMA MicroPortable viable particle counter. The SMA particle counter is located next to the containers being filled. Sequential sampling of 10^3 ft of air onto Trypticase Soy Agar (TSA) plates placed within the SMA unit is accomplished with a new TSA plate challenged every thirty (30) minutes. After incubating the plates at 20-25 °C for 7 days, the results are recorded into the BPCR and reviewed by a Quality Control Unit (QCU) member.

Environmental monitoring of the air for total particles is achieved using a MET One laser particle counter Model A2408 equipped with an eight (8) foot transit tube. During filling operations the MET One probe is placed next to the containers being filled. The sampled volume is 1.0 ft^3 collected over 1 minute. Particle counts ≥0.5μm are recorded at five minute intervals during the filling operation. The raw data are included in the BPCR.

The filling operation is a seamless, four stage process: (1) bulk product is pumped from a sterile holding vessel through sterile Wheaton 3mm ID silicone tubing; the tubing is fitted with a six inch dispensing nozzle that is used to deliver a uniform volume to each container; a dedicated operator fills the containers; (2) a second dedicated operator applies stoppers to the filled containers using sterile forceps, (3) a third dedicated operator applies seals to the filled and stoppered containers, (4) a fourth dedicated operator crimps the vials. All operations are performed under a Class 100 VLF HEPA hood.

Throughout the filling operation a technician is responsible for staging materials, supplying operators with packaging components and removing finished product from the filling table. A microbiologist is responsible for air, surface and personnel monitoring, as well as recording the lot numbers of all packaging components used in the fill in the BPCR. A production supervisor oversees the filling process to ensure adherence to proper procedures and aseptic technique.

At the conclusion of the fill, TSA/Lecithin/Polysorbate-80 touch plates are used to sample the sleeves and gloves of dedicated operators. The plates are incubated for seven days at 30 – 35°C; the results are read and recorded. The QCU is responsible for the analysis of results and for determining if follow-up is required. Deviations from established procedures are documented. All deviations must be justified or the product must be discarded.

Quality Control Testing of Drug Product

The tests performed on drug product produced in final containers are shown in Table 9.4 below. The results of testing two lots of LtSTA containing 0.5 mg/mL of protein are shown in the table.

Table 9.4: Processing Data for Final Drug Product Containing 0.5 mg/mL of protein

Lot Number:	XLtSTA016	XLtSTA017
Protein (mg/mL)	0.51	0.53
Phenol (0.40%w/v)	0.42	0.42
Glycerin (1.0%)	0.87	0.89
NaCl (0.85%)	0.88	0.88
pH (7.0)	6.9	6.9
Safety	Pass	Pass
Potency	Pass	Pass
BSA (ng/mL)	23.46	21.14
Phosphate (mg/mL)	2.61	2.54
Sterility Test	Pass	Pass

STATISTICAL

10. *For the future Phase 3 trial, a general statistical analysis plan (SAP) describing the study objectives, hypotheses (expressed formally with statistical notations and explanations), primary endpoints, design specifications, criteria for declaring study success and statistical analysis methods, should be submitted for CBER review. Note that the SAP is not considered final until it is concurred upon by CBER. We recommend that you not initiate a phase III trials without submitting the SAP, and that it be finalized early on during the comment period. This is to assure that the study design and planned analyses are adequate to meet the stated objectives. Accordingly, failure to submit the SAP until after the study has been completed and just prior to data lockdown will potentially be problematic.*

A Phase III protocol is enclosed (Attachment I). Section 32 of the protocol describes the statistical analyses that will be performed with the data that will be obtained from the three study groups. The statistical plan is attached as a separate document (Attachment J). Please see item 11 below.

CLINICAL

Regarding the final study report (FSR) for study LtSTA-08, revision 03 and 03A, as well as the previously submitted Phase 1 and phase 2 FSRs:

11. *These small studies are more appropriately considered Phase 1 proof of concept studies rather than Phase 2 studies. The data submitted thus far indicate insufficient characterization of the sensitivity, specificity or sensitizing properties of the LtSTA article. This may*

be related in part to insufficient characterization and inconsistent manufacturing of your product. Please comment.

The *Leishmania* skin test has been used and is currently being used as a diagnostic and epidemiological tool by medical professionals and scientists on a world-wide basis. The safety and efficacy of the skin tests has been clearly established, since it was first used by Montenegro. [(2)] The publications included in the References below are a few examples of the many studies that have been conducted with *Leishmania* skin test antigen made from whole promastigotes or lysates of the parasite.

Our initial 2004 study enrolled 32 healthy volunteers without a prior history of leishmaniasis. This study was designed to assess the safety of LtSTA and to gain preliminary evidence on its specificity. According to 21 CFR 312.21(a)(1), a Phase I investigation is the "*initial introduction of an investigational new drug into humans...designed to determine the metabolism and pharmacologic actions of the drug in humans, the side effects associated with increasing doses, and, if possible, to gain early evidence on effectiveness.... The total number of subjects and patients included in Phase I studies varies with the drug, but is generally in the range of 20 to 80.*" We believe that Allermed's Phase I study meets this definition.

A Phase II study conducted in 2007 was a safety and proof of concept efficacy study that enrolled a total of 100 subjects. Dose ranging of LtSTA in the first arm of the study was evaluated in 20 subjects to determine an appropriate dose to use in the second and third arms of the study. A 30 μg dose was selected and that dose was evaluated for safety and efficacy in the remaining two arms of the clinical trial with each arm containing 40 subjects. Sensitivity was evaluated in 40 subjects with a confirmed history of CL; specificity was evaluated in 40 subjects without a confirmed history of CL.

A Phase IIB study was a blinded, placebo controlled trial to evaluate the safety, specificity (false positive reactions), and potential sensitizing properties of 15, 30, and 50 μg intracutaneous doses of LtSTA. Fifty (50) subjects were enrolled in the trial; 41 subjects completed the trial. One of the primary goals of the study was to explore the potential for false positive reactions, *i.e.* specificity of the antigen in people without a previous history of leishmaniasis.

The total number of subjects enrolled to date in these controlled Phase II studies to provide proof-of-concept efficacy and additional safety information is 141. According to 21 CFR 312.21(b), Phase II "*includes the controlled clinical studies conducted to evaluate the effectiveness of the drug for a particular indication or indications in patients with the disease or condition under study and to determine the common short term side effects and risks associated with the drug. Phase II studies are typically well controlled, closely monitored, and conducted in a relatively small number of patients, usually involving no more than several hundred subjects.*" We believe that Allermed's Phase II studies meet the definition described in the regulation.

Based on the previous clinical investigations that have been completed, Allermed intends to conduct a phase III clinical trial in which the safety, sensitivity and specificity of LtSTA will

be evaluated with product containing 50 ug/mL of protein. The rationale for using this dose is based on the following points:

1. A phase I study conducted by Allermed demonstrated that LtSTA containing 20 μg to 120 μg/0.1 mL of Ninhydrin protein could be administered to *Leishmania* naïve human volunteers without causing unsafe reactions.
2. A phase II study conducted by Allermed demonstrated that 30 μg/0.1 mL of LtSTA could be safely administered to human volunteers. Forty volunteers with a history of active cutaneous leishmaniasis within the past 24 months and 40 volunteers with no history of cutaneous leishmaniasis were skin tested with the 30 μg product. Follow up testing of 6 individuals who had histories of leishmaniasis, but who were skin test negative to the 30 μg doses demonstrated that a 50 μg dose elicited a positive DTH response in all 6 volunteers without causing serious adverse events.
3. A phase IIB study conducted by Allermed demonstrated that three repeat doses of 30 μg and 50 μg of LtSTA could be safely administered to *Leishmania* naïve human volunteers. Some study subjects converted from skin test negative to skin test positive with both the 30 μg and 50 μg doses on the third skin test.
4. The results of the phase II study in which six subjects with histories of leishmaniasis failed to react to the 30 μg, but were skin test positive to the 50 μg dose demonstrated the superiority of the 50 μg dose in detecting sensitization in subjects with a history of leishmaniasis. The results of the Phase IIB study in which both the 30 μg and 50 μg doses induced sensitization in some individuals after two skin tests revealed that the potential to sensitize Leishmania naïve individuals was present with both doses. Therefore, the 50 μg dose was selected for a Phase III trial based on its superior performance in detecting sensitization to the parasite.
5. The three clinical studies conducted by Allermed were appropriately labeled as Phase I, Phase II, and Phase IIB as defined in 21 CFR 312.21. It is our opinion that the pathway that has been followed in the clinical development of LtSTA is appropriate for a skin test antigen. We have addressed the issues of safety, dose, sensitivity and specificity with adequate numbers of subjects to provide a basis for conducting a phase III trial. It is expected that further characterization of safety, sensitivity, specificity and sensitization will be provided by the data obtained in a phase III study.

Reports of the clinical trials conducted by Allermed have been submitted to the IND file. Allermed considers these trials to satisfy the requirements of phase1 and phase 2 studies as defined in 21 CFR 312.21 (a)(1) and 21 CFR 312.21(b).

12. *Please clearly state your proposed indication for LtSTA including a description of how this product would be used to manage patients in a clinical setting. This information is essential for the evaluation of the risks and benefits of your product, and to guide clinical development. Please note that your proposed indication should be "for*

the risks and benefits of your product, and to guide clinical development. Please note that your proposed indication should be "for the treatment, prevention, mitigation, cure or diagnosis of a recognized disease or condition, or of a manifestation of a recognized disease or condition…" [21 CFR 201-57(c)(2)], and that a label must be supported by "substantial evidence of effectiveness" [21 CFR 201.57(c)(2)(v)].

INDICATED USE OF LtSTA

LtSTA will be used to detect delayed-type IV cell mediated hypersensitivity following cutaneous infection with *Leishmania major*. The antigen can be used to diagnose active cutaneous leishmaniasis caused *by L. major*. It also can be used to detect delayed-type IV cell mediated immunity in persons with a history of healed cutaneous leishmaniasis caused by *L.major* within the past 24 months.

USE OF LtSTA IN A CLINICAL SETTING

A positive skin test to LtSTA in persons with active lesions characteristic of cutaneous leishmaniasis is presumptive evidence of infection with *L. major*. However, confirmation of the disease must be made by standard laboratory methods involving isolation of the organisms, or by other established laboratory procedures.

Persons suspected of having undiagnosed cutaneous leishmaniasis within the past 24 months who have a positive skin test to LtSTA may have been infected with *L.major*. This information may be relevant in the medical history of the individual.

13. *Once the broader issues of the product indication and the manufacture of your product are addressed, we will be better able to assist you with a clinical development plan.*

Thank you for your interest in assisting us with the development of this important product.

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December 10, 2010

Director
Center for Biologics Evaluation and Research
1401 Rockville Pike
HFM-475, Suite 200 N
Rockville, MD 20852-1448

RE: BB-IND 11822
Annual Report

Dear Director:

This submission is the Annual Report for BB-IND 11822 regarding *Leishmania tropica* Skin Test Antigen (LtSTA) for Cellular Hypersensitivity. This product is being developed under contract W81XWH-09-C-0138-P0001 with the U.S. Army Medical Research and Material Command (USAMRMC).

During the 2010 reporting period, the following actions occurred:

1. A final report for Study LtSTA-08 was submitted to CBER on January 27, 2010. This study was completed on September 27, 2009 and closed out on January 17, 2010 after a final meeting with the principal investigator, Dr. Donald Brandon, and the research staff who assisted him in conducting this clinical trial. A synopsis of the study was included in the 2009 Annual Report.

2. A request for an end-of-phase II Type B meeting was submitted to CBER on February 17, 2010. In response to this request CBER asked Allermed to provide a summary of the current status of IND 11822. This information was sent to CBER on March 4, 2010 and March 8, 2010. After reviewing the March submissions, CBER sent a written response dated May 7, 2010 to Allermed requesting additional information.

3. A final report for an unexpected adverse event that occurred in Study LtSTA-08 was submitted to CBER on April 15, 2010. This event involved abnormal urinary findings in the exit laboratory tests of a nineteen year old male participant. In a follow up contact with the principal investigator made on December 7, 2010, Allermed was informed that this individual had not experienced a recurrence of the event and the urinary condition associated with the adverse event was considered to be stable.

4. On November 4, 2010, Allermed submitted the information requested by CBER in the May 7, 2010 letter. During the five months between the May 7, 2010 correspondence from CBER and the November 5, 2010 reply, Allermed revalidated product processing and the testing procedures for product containing 0.05 mg per mL. The 0.05 mg per mL (50 µg/0.1mL) concentration was established as the optimum dose of the skin test antigen from the results of a phase II study (LtSTA-06) and a phase IIB study (LtSTA-08) conducted by Allermed.

Please let us know if you have any questions concerning this report. We shall be pleased to respond by providing additional information regarding the work that has been done on this product.

Sincerely,

H.S. Nielsen, Jr., Ph.D.
President

HSN:sp
Enclosures:
1. Biomed IRB Continual Approval Notification, Exp. 02/13/2010.
2. Biomed IRB Close-out Notification date January 18, 2010.

BioMed·IRB

IRB Meeting Date: February 4, 2009 **Expiration Date:** February 13, 2010

BIOMED IRB CONTINUAL APPROVAL NOTIFICATION

Study Title: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 15μg, 30μg and 50μg Intracutaneous Doses of *Leishmania tropica* Skin Test Antigen (LtSTA) In Adult Volunteers without a History of Exposure to *Leishmania spp.*

Sponsor: Allermed Laboratories, Inc.

Protocol Number: LTSTA-08

Protocol Dates: Revision 0 dated February 1, 2008
Revision 02 dated June 17, 2008
Revision 03 dated July 21, 2008
Revision 03 (Responsibilities Changes) dated July 21, 2008
Addendum to Protocol dated November 17, 2008

Principal Investigator: Donald M. Brandon, MD

Approved Facilities: California Research
Foundation
2800 Third Avenue
San Diego, CA 92103-6204

BioMed IRB has approved the above referenced study as having satisfied the criteria for continuing research at the February 4, 2009 meeting. This approval is effective from February 13, 2009.

The IRB committee has determined that the risk assessment for this study is More than Minimal. The IRB has determined that continuing review of this study will occur annually.

Approximately thirty days before February 13, 2010, you will be required to complete a Continuing Review Report Form. Continual review is the responsibility of the Principal Investigator. If you do not receive this form, please contact the IRB office immediately. The Continual Review Report Form must be received by the due date to allow ample time for ongoing review before the study's expiration date.

IRB approval is granted conditional on your adherence to the following requirements:

- The information submitted to the IRB is true and correct.
- Research will be conducted in accordance with the approved protocol.
- All materials used to recruit study subjects must be pre-approved by the IRB.
- Additional safeguards will be followed when vulnerable subjects, such as children or minors, are participants in the study.

The investigator agrees to report the following information to the IRB:

- Serious Adverse Events occurring at your site should be reported within ten (10) calendar days from the date of discovery by the investigator.
- Serious Adverse Events (IND Safety Reports) occurring at other sites should be reported no later than thirty (30) days from the date of discovery.
- Any changes in the research activity (i.e. changes in study staff, facility etc.) should be reported promptly. In addition, the investigator will not make any changes in the research without the IRB's approval, except when necessary to eliminate apparent immediate hazards to study subjects.
- Any other unanticipated problems involving risks to study subjects.

BioMed IRB is comprised of a diverse group of individuals in accordance with the Federal Regulations and the International Conference on Harmonization guidance for Good Clinical Practice. BioMed IRB follows written procedures for performing review, documenting meeting minutes, disclosure of member conflict of interest prior to deliberation or voting, as well as the retention of all records containing research materials as required by the Code of Federal Regulations (21CFR parts 50 and 56; and 45 CFR part 46).

On behalf of the BioMed IRB, I certify that the information contained in this letter is true and correct as verified by the minutes and records of the BioMed IRB.

Please keep a copy of the continual review material, as well as a copy of this letter, in your files for future reference. Should you have questions or concerns, please do not hesitate to contact this office.

Sincerely,

Authorized Signature	Human Research Protection Manager Title
Jeremy Markovich Printed Name	February 4, 2009 Date

CC: H.S. Nielsen, Jr., PhD, Allermed Laboratories, Inc.

BioMed•IRB

January 18, 2010

Dr. Donald M. Brandon
California Research Foundation
2800 Third Avenue
San Diego, CA 92103-6204

RE: BIOMED IRB SITE CLOSURE NOTIFICATION

Study Title: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 30µg Intracutaneous Doses of *Leishmania tropica* Skin Test Antigen (LtSTA) In Adult Volunteers Without a History of Exposure to *Leishmania spp.*

Sponsor: Allermed Laboratories, Inc.

Protocol Number: LTSTA-08

Dear Dr. Brandon:

BioMed IRB has received your closure information for the above referenced study. Your report indicates that the study has closed at your site and that all enrollment or research related activities, as stated in the protocol, have been completed. According to 45 CFR 46.115, the study records will be placed in our closed files and remain there for an additional three years. You are no longer required to submit any regulatory documentation to this office.

BioMed IRB is comprised of a diverse group of individuals in accordance with the Federal Regulations and the International Conference on Harmonization guidance for Good Clinical Practice. BioMed IRB follows written procedures for performing review, documenting meeting minutes, disclosure of member conflict of interest prior to deliberation or voting, as well as the retention of all records containing research materials as required by the Code of Federal Regulations (21CFR parts 50 and 56; and 45 CFR part 46).

On behalf of the BioMed IRB, I certify that the information contained in this letter is true and correct as verified by the minutes and records of the BioMed IRB.

Thank you for your compliance and please do not hesitate to contact this office if we can be of further assistance.

Christal Santos

Authorized Signature	Administrator Title
Christal Santos Printed Name	January 18, 2010 Date

CC: H.S. Nielsen, Jr., PhD, Allermed Laboratories, Inc.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
1401 Rockville Pike
Rockville, MD 20852-1448

March 17, 2011

Our Reference: IND 11822

Allermed Laboratories, Inc.
Attention: Dr. Stuart Neilsen, Jr.
7203 Convoy Court
San Diego, CA 92111

Dear Dr. Neilsen:

We have reviewed the information provided in your November 10, 2010, submission to your **Investigational New Drug Application (IND)** for "*Leishmania tropica* Soluble Skin Test Antigen (LtSTA)". This submission contains your response to the FDA letter dated May 7, 2010. Please note that the following comments pertain to the primary issues regarding further development of LtSTA identified at this time, but should not be considered a comprehensive, all-inclusive review of all material submitted in this amendment. We have the following comments, questions, and requests for additional information:

The following comments are regarding the clinical section of the IND:

1. In response to our question regarding the proposed indication of LtSTA (Question #12 of our IR letter dated May 7, 2010), you stated the following:

   INDICATED USE OF LtSTA
   LtSTA will be used to detect delayed-type IV cell-mediated hypersensitivity following cutaneous infection with Leishmania major. The antigen can be used to diagnose active cutaneous leishmaniasis caused by L. major. It can also be used to detect delayed-type IV cell mediated immunity in persons with a history of healed cutaneous leishmaniasis caused by L. major within the past 24 months.

   USE OF LtSTA IN A CLINICAL SETTING
   A positive skin test to LtSTA in persons with active lesions characteristic of cutaneous leishmaniasis is presumptive evidence of infection with L. major. However, confirmation of the disease must be made by standard laboratory methods involving isolation of the organisms, or by other established laboratory procedures.

Persons suspected of having undiagnosed cutaneous leishmaniasis within the past 24 months who have a positive skin test to LtSTA may have been infected with L. major. This information may be relevant in the medical history of the individual.

The proposed Phase 3 study does not include sufficient evaluation of LtSTA responses in Tunisian subjects without prior history of leishmaniasis (or with history of healed CL previous to the past 24 months) to support the proposed indication specific to persons with healed cutaneous leishmaniasis (CL) caused by *L. major* within the past 24 months. Furthermore, it is unclear how LtSTA would be applied to the clinical management of persons with suspected healed CL.

Your clinical development program for LtSTA, including the proposed pivotal Phase 3 study (LtSTA-11), may at best provide data to support interpretation of a positive response to LtSTA (> 5 mm) as a presumptive diagnosis, pending confirmatory testing by standard laboratory methods, of active cutaneous leishmaniasis (CL) due to *L. major* in otherwise healthy Tunisian adults with positive response to two anergy controls, no previous exposure to *Leishmania spp.* other than *L. major*, and no previous exposure to LtSTA or other *Leishmania* skin test or vaccine.

Other issues pertinent to the effectiveness of this product for diagnosis of active CL include the following:

a. Due to the possibility that sensitization occurs with the intended LtSTA dose (positive responses > 5 mm in three non-endemic subjects following the 3rd dose of 30 μg or 50 μg LtSTA in the Phase 2b study); this product would be restricted to one-time use in any patient.

b. The pre-test probability of active CL among Tunisian adults with lesions suspicious for this infection will likely be quite high. Since a positive response to LtSTA will require confirmatory testing using standard methods, most patients with suspicious lesions who are administered LtSTA (assuming a positive response) will undergo standard diagnostic procedures. In cases where there is clinical suspicion for active CL but negative response to LtSTA (potentially due to anergy or other obscuring medical condition), standard diagnostic procedures will likely be conducted as well. Therefore, it is unclear how LtSTA will enhance the diagnosis of CL compared to current methods.

c. You suggest in your proposed Phase 3 protocol introduction that a FDA-licensed *Leishmania* skin test may be useful for the diagnosis of CL caused by *L. major* among U.S. military personnel and travelers with exposure to *L. major*-endemic areas in the Middle East. However, to potentially support an indication of this type, the clinical development plan would require substantial changes to the design of the proposed Phase 3 study, as well as one or more additional Phase 2

studies evaluating the sensitivity and specificity of LtSTA among U.S. residents with skin lesions and exposure histories suspicious for CL due to *L. major*.

Given the limitations outlined above, several of the indications you have proposed are not likely to be supported by substantial evidence of effectiveness necessary for labeling of biological products (see 21 CFR 201.57(c) (2) (v)). Please clarify the intended target population(s) for this product as well as how you propose to demonstrate effective performance of a clinically significant function in the target population(s).

2. The product appears likely to contain immunomodulatory component(s) based on sensitization observed among some naïve subjects. In light of this observation as well as the uncertain identity of suspected immunomodulatory components, we recommend that any future clinical studies incorporate an extended follow-up period of six months in order to assess specific outcomes, including: 1) frequency of adverse events; 2) clinical course of subjects with confirmed active CL; and 3) clinical manifestations of *Leishmania* infections occurring in subjects who have previously received LtSTA.

3. Please note that the above comments do not constitute a formal comprehensive review of your proposed Phase 3 protocol. We strongly recommend that you postpone initiation of any further clinical studies until we reach agreement with you regarding the sufficiency of a revised protocol to meet its stated objectives.

The following comments are regarding the CMC section of the IND:

4. With regard to your overall product development and testing plans, we note that in your study designated "Phase 2b," in which subjects were administered three repeated doses of LtSTA, detectable skin test reactions developed in over 15% of all naive subjects. These unexpected observations imply that LtSTA administered intradermally, especially at doses of 30 µg and 50 µg, is highly immunogenic. Proteins in isolation are generally poorly immunogenic. The sensitization results noted above imply the presence of protein(s) in highly immunogenic forms (e.g., lipidated or aggregated), and/or complexed with other *Leishmania*-derived component(s) that effectively serve as an adjuvant. This may be consistent with the nature of the LtSTA product, which is comprised of a soluble fraction obtained after homogenizing promastigotes with abundant lipophosphoglycans (LPG), glycoinositol phospholipids, and membrane glycoproteins. Therefore, substantial product characterization is warranted.

With regard to product characterization and testing, licensure of LtSTA will require that you implement a product development plan that appropriately characterizes the LtSTA product, demonstrates consistency of manufacture, and provides physicochemical testing strategies to address the following considerations:

5. Although we recognize that diagnostic products are exempted from endotoxin testing, it is to your advantage to incorporate this assessment because of the suspected presence of an immunomodulatory component.

6. While the use of phenol is acceptable for multidose vials, in this case its addition is problematic due to the possibility of protein denaturation and aggregation that would impact the physical form of the final product. Protein aggregation is one possible explanation for the ability of LtSTA to induce sensitization. Thus, characterization of the final product with excipients included will be necessary. Alternatively, you may consider the use of single dose vials and removal of phenol.

7. We note that you have produced 17 lots to date, but the disposition and use of each lot is not clear. Please provide a tabular summary of information regarding the lots manufactured to date, to include whether each has been used in a clinical trial (and if so, identify the study number) and if it has been evaluated in a stability protocol. Please note that lots placed into stability testing should be those used in clinical testing. Furthermore, until you have accumulated additional characterization data for your product and demonstrated that you can evaluate adequately consistency among batches, only one lot of product should be used within any clinical study; the use of multiple lots during repeat dose studies or for different dilution series within a study may confound interpretation of clinical data.

8. Because of the clinical study observations, as well as the need to demonstrate consistency of manufacture by appropriate laboratory and clinical testing, we reiterate the need to perform further physicochemical characterization of the bulk as well as final product. It is likely that development of additional lot release tests will be needed, as indicated by the results of product characterization. We recommend that at a minimum, these efforts include the following:

    a. Determination of the identities of at least some of the major protein components of the bulk product, and examination of the physical state of the major components in the final product.

    b. Determination of the relative quantities of the major protein components of the bulk and final products, e.g., using scanning densitometry.

    c. Establishment of specifications for the relative quantities of the major protein components of the final product, i.e., range of relative proportions of protein bands designated as 'major.'

    d. Determination of the quantities of carbohydrate and lipid components, and of carbohydrate and lipid modifications of protein components. These results may indicate the need for identifying individual components.

e. Comparison of the protein sequences of the major components to publicly available genomic and EST sequences of humans, other *Leishmania* species, and other potentially cross-reacting species.

9. We note that prolonged heat treatment (which might reasonably be expected to adversely affect potency) apparently did not change the outcome in your current potency assay. You state that the potency assay is under revision and that you plan to report a relative potency value. We encourage this approach, but also advise that further development of the proposed potency assay is required to ensure that the resulting format reliably distinguishes between potent, sub-potent, and suprapotent lots. As your experience with the revised assay continues, a range of error for the relative potency value should be established.

In reply to this letter, we recommend that you restate each item and follow it with your explanation, clarification, or comment. Use of this format helps to organize the relevant information and provides a self-contained document that facilitates future reference.

If you have any questions, please contact Dr. Joseph Temenak at (301) 796-2640.

Sincerely yours,

Wellington Sun, M.D.
Director
Division of Vaccines and
Related Products Applications
Office of Vaccines
Research and Review
Center for Biologics
Evaluation and Research

The following comments are regarding the clinical section of the IND:

FDA COMMENT:

1. *In response to our question regarding the proposed indication of LtSTA (Question #12 of our JR letter dated May 7, 2010), you stated the following:*

   INDICATED USE OF LtSTA

   LtSTA will be used to detect delayed-type IV cell-mediated hypersensitivity following cutaneous infection with Leishmania major. The antigen can be used to diagnose active cutaneous leishmaniasis caused by L. major. It can also be used to detect delayed-type IV cell mediated immunity in persons with a history of healed cutaneous leishmaniasis caused by L. major within the past 24 months.

   USE OF LtSTA IN A CLINICAL SETTING

   A positive skin test to LtSTA in persons with active lesions characteristic of cutaneous leishmaniasis is presumptive evidence of infection with L. major. However, confirmation of the disease must be made by standard laboratory methods involving isolation of the organisms, or by other established laboratory procedures.

   Persons suspected of having undiagnosed cutaneous leishmaniasis within the past 24 months who have a positive skin test to LtSTA may have been infected with L. major. This information may be relevant in the medical history of the individual.

The proposed Phase 3 study does not include sufficient evaluation of LtSTA responses in Tunisian subjects without prior history of leishmaniasis (or with history of healed CL previous to the past 24 months) to support the proposed indication specific to persons with healed cutaneous leishmaniasis (CL) caused by L. major within the past 24 months. Furthermore, it is unclear how LtSTA would be applied to the clinical management of persons with suspected healed CL.

Your clinical development program for LtSTA, including the proposed pivotal Phase 3 study (LtSTA-11), may at best provide data to support interpretation of a positive response to LtSTA (> 5 mm) as a presumptive diagnosis, pending confirmatory testing by standard laboratory methods, of active cutaneous leishmaniasis (CL) due to L. major in otherwise healthy Tunisian adults with positive response to two anergy controls, no previous exposure to Leishmania spp. other than L. major, and no previous exposure to LtSTA or other Leishmania skin test or vaccine.

The protocol that was submitted with our November 10, 2010 correspondence was intended to support the use of LtSTA as a screening test for past exposure to *Leishmania major*. Due to the one-time use of the product, its value as a screening tool for persons that live in or periodically enter endemic areas multiple times is limited. For this reason, Allermed intends to change the indicated use of LtSTA. Future development of the product will be based on the use of LtSTA as a diagnostic test for cutaneous leishmaniasis (CL).

Rationale for the Indication: In a dose-response phase 2 study conducted by Allermed involving persons with active cutaneous leishmaniasis (CL) the incidence of positive skin tests was 100%. Four cohorts of study subjects were skin tested with either 10 mcg, 20 mcg, 40 mcg or 80 mcg of LtSTA. Each cohort consisted of five adult volunteers. Positive induration responses to LtSTA after 48 hours were observed in all individuals in each dose group.

Based on linear regression analysis of the dose-response data, a dose of 30 mcg was selected to test a second cohort of adult volunteers with a history of active CL within the past 24 months. Positive skin tests were observed in 34/40 (85%) of these individuals. In a follow-up study using a 50 mcg dose, the 6 persons with negative skin tests to the 30 mcg dose reacted with positive skin tests to the 50 mcg dose.

In a third cohort of study subjects without a history of CL, 98% (39/40) individuals were skin test negative to LtSTA.

These observations provide strong evidence that LtSTA-

(1) Can detect delayed-type hypersensitivity (DTH) in persons with active CL caused by *L. major,*

(2) Can detect DTH in persons without active CL, but with a history of CL within the past 24 months caused by *L. major.*

(3) Does not elicit a positive DTH response in persons that do not have a history of CL caused by *L. major.*

To provide appropriate power for the analysis, 60 adult volunteers with *L. major* active CL in various stages of disease development will be tested with LtSTA on a blinded basis, using appropriate positive and negative controls. A second cohort of 30 *Leishmania* naïve subjects with lesions that might be misdiagnosed as CL will be skin tested with LtSTA. A third group of 60 *Leishmania* naïve subjects without cutaneous lesions will be tested as negative controls against the other two cohorts. The study is intended to (1) show that the sensitivity of LtSTA in detecting *L. major* as a cause of cutaneous lesions is at least 80% at the 95% CI and (2) the specificity of LtSTA in distinguishing cutaneous lesions caused by *L. major* from cutaneous lesions caused by other agents or conditions is at least 85% at the 95% CI, and is not significantly different from the sensitivity observed in *Leishmania* naïve subjects without lesions.

Clinical Importance: The clinical expression of CL caused by *L. major* can vary from a small pimple-like sore to large ulcers. At the present time, the diagnosis of CL is based on demonstrating or culturing the parasite in the clinical laboratory from exudate or tissue samples. PCR technology also may be used. However, under circumstances in which neither of these

procedures is available, such as field conditions experienced by military personnel, or in cases in which low numbers of parasites are present, a positive skin test to LtSTA could be used to establish a diagnosis of CL.

Use of LtSTA in a non-endemic population: Under the indication described above, the test could be administered to persons returning from a *Leishmania* endemic area who have undiagnosed cutaneous lesion(s).

Use of LtSTA in an endemic population: Under the indication described above, the test could be administered to persons with undiagnosed cutaneous lesions.

FDA COMMENT:

Other issues pertinent to the effectiveness of this product for diagnosis of active CL include the following:

1.a. Due to the possibility that sensitization occurs with the intended LtSTA dose (positive responses > 5 mm in three non-endemic subjects following the 3rd dose of 30 μg or 50 μg LtSTA in the Phase 2b study); this product would be restricted to one-time use in any patient.

As a diagnostic test for CL caused by *L. major*, LtSTA can be limited to one-time use. As such, the sensitizing properties of the skin test become less important. Initially, Allermed intended LtSTA to be a multiple-use product to monitor the sensitivity of persons redeployed to endemic areas. Allermed was aware of the possibility that LtSTA might be sensitizing from published reports of other *Leishmania* skin test products. [(1)] The sensitizing capacity of LtSTA was suspected from observations involving two naïve individuals in Allermed's phase 1 safety trial. These individuals developed a delayed-type induration response approximately two weeks after being skin tested with 120 mcg of the product. We interpreted this finding as a delayed-type inflammatory response to the *Leishmania* antigen retained in the skin, which reacted with lymphocytes that had developed sensitivity to LtSTA from the initial skin test. In 2001, Jose et al. [(1)] reported that conversion from negative to positive delayed-type hypersensitivity occurred after skin tests with *L. amazonensis* antigen in three of nine (3/9) naïve subjects 30 days after a single skin test had been administered. In the same cohort of study subjects, six of the nine (6/9) subjects converted from a negative to positive DTH response after 90 days. The antigen used was a soluble lysate of the parasite. The same investigators also studied DTH conversion after a single skin test with a particulate antigen made from sonicated promastigotes. In this group of study subjects, 42% reacted to a second skin test with a positive induration response after 30 days, and 50% of the subjects were positive to a second skin test after 90 days. The soluble antigen contained 0.4% phenol as a preservative. The particulate antigen was preserved with 1:10,000 Merthiolate.

1.b. The pre-test probability of active CL among Tunisian adults with lesions suspicious for this infection will likely be quite high. Since a positive response to LtSTA will require confirmatory testing using standard methods, most patients with suspicious lesions who are administered LtSTA (assuming a positive response) will undergo standard diagnostic procedures. In cases where there is clinical suspicion for active CL but negative response to LtSTA (potentially due to anergy or other obscuring medical condition), standard diagnostic procedures will likely be conducted as well. Therefore, it is unclear how LtSTA will enhance the diagnosis of CL compared to current methods.

The use of *Leishmania* skin test as a diagnostic test for active CL is widely practiced in endemic areas; the correlation between a positive delayed-type skin test and existence of the disease is high. This was observed in Allermed's phase 2 study in which 20/20 persons with active CL had positive skin tests to LtSTA. Allermed intends to show that in persons with active CL the sensitivity to a 50 mcg dose of LtSTA is ≥80% at the 95% CI. Confirmation of the disease will be made by culturing the parasite from the lesion. The role of anergy as a factor can be minimized by the use of companion DTH antigens. Current laboratory methods are not always available in the field to establish a diagnosis of CL. Under these conditions, a positive LtSTA skin test would be diagnostic of CL infection.

We acknowledge the possibility that the LtSTA skin test can be negative and clinical observations positive for CL. However, we do not anticipate that this will happen, based on the results of Allermed's phase 2 Study and the experience of clinicians practicing in endemic areas where it is widely accepted that a positive skin test to *Leishmania* antigen is diagnostic for active CL (communication from Dr. Afif Ben Salah, Pasteur Institute, Tunis, Tunisia).

1.c. You suggest in your proposed Phase 3 protocol introduction that a FDA-licensed Leishmania skin test may be useful for the diagnosis of CL caused by L. major among U.S. military personnel and travelers with exposure to L. major-endemic areas in the Middle East. However, to potentially support an indication of this type, the clinical development plan would require substantial changes to the design of the proposed Phase 3 study, as well as one or more additional Phase 2 studies evaluating the sensitivity and specificity of LtSTA among U.S. residents with skin lesions and exposure histories suspicious for CL due to L. major.

Given the limitations outlined above, several of the indications you have proposed are not likely to be supported by substantial evidence of effectiveness necessary for labeling of biological products (see 21 CFR 201.57(c) (2) (v)). Please clarify the intended target population(s) for this product as well as how you propose to demonstrate effective performance of a clinically significant function in the target population(s).

We believe that the indicated use of LtSTA meets the 21 CFR 201.57 (c) (2) (v) regulations for a biological product. Identifying delayed-type hypersensitivity to an etiologic

agent in which cellular hypersensitivity to the organism is a sequela of the infection is a diagnostic test.

We intend to include persons with various types of skin lesions in our clinical trial. Diseases that might be misdiagnosed as cutaneous leishmaniasis include Herpes zoster, impetigo, diabetic ulcers, cutaneous T cell lymphoma, bacterial infection of the skin, arterial ulcer in drepanocytosis, pemphigus vegetans, basal cell carcinoma, carbuncle, post traumatic ulcer, pyogenic granuloma and cutaneous *Trypanosoma* infection.

2. *The product appears likely to contain immunomodulatory component(s) based on sensitization observed among some naïve subjects. In light of this observation as well as the uncertain identity of suspected immunomodulatory components, we recommend that any future clinical studies incorporate an extended follow-up period of six months in order to assess specific outcomes, including: 1) frequency of adverse events; 2) clinical course of subjects with confirmed active CL; and 3) clinical manifestations of Leishmania infections occurring in subjects who have previously received LtSTA.*

It is possible to follow study subjects in the U.S. for as long as 6 months after the administration of LtSTA. However, in endemic areas, a 6 month follow up of study volunteers may not be possible. This will be discussed with the principal investigator involved with volunteers who reside in the endemic area of the study. We would like you to be more specific in describing what information will be required in following the clinical course in subjects with active CL and the clinical manifestations of *Leishmania* infections occurring in persons who have previously received LtSTA

FDA COMMENT

3. *Please note that the above comments do not constitute a formal comprehensive review of your proposed Phase 3 protocol. We strongly recommend that you postpone initiation of any further clinical studies until we reach agreement with you regarding the sufficiency of a revised protocol to meet its stated objectives.*

We understand that the comments included in FDA letter dated March 17, 2011 do not represent a formal review of the phase 3 protocol that was submitted. We also believe that agreement between FDA and Allermed on the design of a phase 3 trial is necessary before a trial begins.

FDA COMMENT

The following comments are regarding the CMC section of the IND:

4. *With regard to your overall product development and testing plans, we note that in your study designated "Phase 2b," in which subjects were administered three repeated doses of LtSTA, detectable skin test reactions developed in over 15% of all naïve subjects. These unexpected observations imply that LtSTA administered intradermally, especially at doses*

of 30 mcg and 50 mcg, is highly immunogenic. Proteins in isolation are generally poorly immunogenic. The sensitization results noted above imply the presence of protein(s) in highly immunogenic forms (e.g., lipidated or aggregated), and/or complexed with other Leishmania-derived component(s) that effectively serve as an adjuvant. This may be consistent with the nature of the LtSTA product, which is comprised of a soluble fraction obtained after homogenizing promastigotes with abundant lipophosphoglycans (LPG), glycoinositol phospholipids, and membrane glycoproteins. Therefore, substantial product characterization is warranted.

In characterizing the drug substance and the final drug product we have identified the dominant proteins that are present in LtSTA using SDS-Page, scanning densitometry and mass spectrometry. The presence of glycoproteins and lipoproteins in the crude lysate has been investigated using staining procedures that are specific for these compounds.

You point out that the promastigotes lysate may contain an abundance of lipophosphoglycans, glycoinositol phospholipids and membrane glycoproteins. From the published literature, it is clear that a variety of compounds, including those you mentioned, are present on the surface and internal structure of the *Leishmania* promastigote. These compounds have been found in several *Leishmania* species and have been shown to exhibit extensive glycosylation with phosphoglycan chains. [(2)] A major membrane surface glycoprotein, referred to as p63, has been found in *L. donovani, L. major, L. tropica*, *L. mexicana*, and *L. braziliensis*. In the native state, p63 is identified at 58 kDa, but in a reduced form it migrates to 63-65 kDa. Soteriadou et al. [(3)] postulated that the 58 kDa and 63 kDa components are the glycoprotein identified as p63. Based on the work of Khabiri et al. [(4)], it is possible that the 58 kDa material is a major contributor to the cellular hypersensitivity response to *Leishmania*. However, other compounds might also be involved as evidenced by a study which demonstrated the importance of a 30 kDa compound in sensitizing mice to *L. amazonensis* [(5)]. From the published literature, it is possible that p63 or the 58 kDa component of the *Leishmania* lysate might represent a protein(s) or protein-conjugate(s) which, by skin test, could detect sensitization from infection with *Leishmania*. However, it is reasonable to assume that other components of the lysate might also be important, such as the 30 kDa component [(5).] Guinea pigs studies conducted at Allermed demonstrated a high degree of cross reactivity of LtSTA in animals sensitized to L. *major* and *L. infantum*, indicating that *L. tropica, L. major* and *L. infantum* share common antigenic properties which could be related to a component, such as p63 discussed above.

In the SDS-PAGE studies reported in our IND application, polyacrylamide gels were stained with silver nitrate. Using silver nitrate, bands were observed at 70 kDa, 51 kDa, 25 kDa, 20 kDa and 8 kDa. More recently, using Coomassie Blue Stain, bands at 67 kDa, 58 kDa, 30 kDa, 20 kDa and 8 kDa have been observed. The band at 67 kDa is visible only in recently manufactured lots of LtSTA and disappears within months during storage at 1-5 °C. The 67 KDa band is believed to be unrelated to the potency of the product. In comparing gels stained with silver nitrate and Coomassie Blue, the (70 kDa and 67 kDa bands), the (51 KDa and 56 kDa

bands), and the (25 kDa and 30 kDa bands) appear to represent the same components. One published study (2) supports the immunogenicity of the 58 kDa component which is present as a major component in LtSTA as shown by scanning densitometry (discussed in item 6). Allermed has identified the proteins in the 67 kDa, 58 kDa, 30 kDa, 20 kDa and 8 KDa regions of Coomassie Blue stained gels.

FDA COMMENT

With regard to product characterization and testing, licensure of LtSTA will require that you implement a product development plan that appropriately characterizes the LtSTA product, demonstrates consistency of manufacture, and provides physicochemical testing strategies to address the following considerations:

5. *Although we recognize that diagnostic products are exempted from endotoxin testing, it is to your advantage to incorporate this assessment because of the suspected presence of an immunomodulatory component.*

Allermed has conducted studies on the presence of endotoxin in LtSTA. Work that has been done to using gel lysate test indicates that LtSTA does not contain endotoxin. This finding will be validated.

FDA COMMENT

6. *While the use of phenol is acceptable for multidose vials, in this case its addition is problematic due to the possibility of protein denaturation and aggregation that would impact the physical form of the final product. Protein aggregation is one possible explanation for the ability of LtSTA to induce sensitization. Thus, characterization of the final product with excipients included will be necessary. Alternatively, you may consider the use of single dose vials and removal of phenol.*

We have found no evidence that phenol is responsible for sensitization. The study of Jose et al. [(1)] showed that *Leishmania* skin test antigen preparations preserved with 0.4% phenol and 1:10,000 parts Merthiolate were both sensitizing. We believe that sensitization is dependent upon the amount of *Leishmania* antigen present in the skin test solution. For example, (a) sensitization occurred in two of eight (25%) naïve subjects after a single skin test with a 120 mcg dose of LtSTA in Allermed's phase 1 safety trail, (b) sensitization occurred between the second and third skin test in one of ten (10%) naïve subjects that received a skin test dose of 50 mcg in Allermed's phase 2B trial, and (c) sensitization occurred between the second and third skin test in one of twenty-three (4%) naïve subjects that received a dose of 30 mcg LtSTA in Allermed's phase 2B trial. In all instances, the amount of phenol in the skin test product was 0.4%. The percentage of persons who became sensitized from the skin test increased as the dose of antigen administered increased.

To more fully evaluate the effects of phenol on the sensitizing properties of LtSTA, naïve guinea pigs were skin tested with LtSTA that was prepared with and without 0.4% phenol. One set of six hairless guinea pigs (HA-HO IAF) was skin tested with 50 mcg doses of LtSTA containing 0.4% phenol. A second set of six guinea pigs (HA-AO IAF) was skin tested with 50 mcg doses of LtSTA prepared without phenol. After 24 hours, each group of animals was observed for induration at the skin test sites; none was observed in either animal group. One week later, a second skin test was administered with the two LtSTA preparations. After 24 hours, guinea pigs in both groups showed diffuse inflammatory reactions at the test sites, but no differences were detected in the response to the phenolated and non-phenolated LtSTA. One week later, animals that initially received phenolated LtSTA were skin tested with Allermed's 50 mcg internal reference standard. This was done to see if the reactions observed in these animals could be elicited with another LtSTA preparation containing 0.4% phenol. After 24 hours, a similar inflammatory reaction was observed. On week six after the first skin test, the two groups of animals were tested a third time with the original phenolated and non-phenolated LtSTA preparations. After 24 hours, the inflammatory response appeared to be the same in both sets of animals. However, the reactions were smaller, less diffuse, and morphologically similar to the skin test response observed following Allermed's sensitization protocol involving the subcutaneous injection of *L. tropica* antigen with Freud's adjuvant.

Based on the findings of this study, the inflammatory response to LtSTA containing 0.4% phenol could not be distinguished from the response to LtSTA prepared without phenol. The sensitizing capacity of the two preparations appeared to be the same.

Protein characterization of phenolated and non-phenolated LtSTA

Phenolated and non-phenolated LtSTA were analyzed by SDS-PAGE , densitometry and mass spectrometry. The proteins present in the 8 kDa, 20 kDa, 30 kDa, 56/58 kDa and 67 kDa bands were determined. No major differences were detected in the phenolated and non-phenolated samples by these procedures. The phenolated product contained all of the proteins that were present in the non-phenolated product, as well as two additional proteases (LmjF.33.1610 and LmjF.05.0960). Over all, phenol did not appear to have a negative effect on the lysate. These data are reported in Attachment 1.

FDA COMMENT

7. *We note that you have produced 17 lots to date, but the disposition and use of each lot is not clear. Please provide a tabular summary of information regarding the lots manufactured to date, to include whether each has been used in a clinical trial (and if so, identify the study number) and if it has been evaluated in a stability protocol. Please note that lots placed into stability testing should be those used in clinical testing.*

Furthermore, until you have accumulated additional characterization data for your product and demonstrated that you can evaluate adequately consistency among batches, only one lot of product should be used within any clinical study; the use of multiple lots during repeat dose studies or for different dilution series within a study may confound interpretation of clinical data.

In our submission dated November 5, 2010, we listed the properties of 17 lots of LtSTA. These lots were selected because they were comparable in terms of the data that could be compared lot to lot. Other lots have been manufactured over the course of LtSTA development. A full accounting of all lots manufactured follows:

Lots XLtSTA001, XLtSTA002 and XLSTA003 were the initial lots of LtSTA manufactured for the purpose of stability assessment. These lots were included in the IND submission and were monitored for stability for 5 years. The lots were manufactured at a Ninhydrin protein concentration of 0.6 mg/ml. The decision to make the product at 0.6 mg/mL was based on a review of published literature. Previously used skin test antigens of *Leishmania* contained approximately 0.3 mg/ml of protein measured by the bicinchoninic acid (BCA) method. Studies at Allermed demonstrated that protein samples measured by the Ninhydrin method contained approximately 2X the amount measured by the BCA procedure, due to differences in the methodology. Because of the presence of phenol in LtSTA, it was not possible to measure protein by BCA. Therefore, the Ninhydrin method was used with a target concentration of 2X 0.3 mg/mL or 0.6 mg/mL.

Lot XLtSTA004 was aborted due to a manufacturing mistake which resulted in a high phenol concentration in the product. This lot was discarded.

Lot XLtSTA005 was formulated with several different buffers to determine effects of pH on the stability of the product.

Source material for Lot XLtSTA006 was divided into three sub-lots labeled as XLtSTA006A, XLtSTA006B and XLtSTA006 at concentrations of 0.6 mg/mL and 1.2 mg/ml. The three sub-lots were used in process development, as were Lots XLtSTA007, XLtSTA008. These lots were not retained after all testing had been completed and the data recorded.

Lot XLtSTA009 was a development lot that was placed on test for 12 months to assess the stability of product formulated with gamma irradiated BSA. Lots manufactured prior to lot XLtSTA009 had been manufactured with BSA that had been heat inactivated. This change in the manufacturing process did not alter the growth of promastigotes and provided another level of insurance against infectious agents in the BSA. In both heat inactivated and gamma irradiated sera, the material was obtained from cattle that were considered to be free of BSE. Following the 12 month stability evaluation, this lot was discarded.

Lot LtSTA010 was a process development lot. It was not used after testing and recording of data for the finished product.

Lot 11 was placebo manufactured for Allermed's phase 1 clinical trial.

Lot XLtSTA012 was manufactured at a concentration of 1.2 mg/mL for use in Allermed's phase 1 clinical trial. This lot was diluted at the clinical site by study personnel. Based on instruction prepared by Allermed, the lot was diluted to 0.20 mg/mL, 0.40, and 0.80 mg/mL. In addition to lot-release testing, this lot was tested for sterility, potency and Ninhydrin protein before/after the phase 1 trial (Sterility: pass/pass, Potency: 12 mm/ 11 mm, Protein: 1.15/1.08 mg/mL).

Lot XLtSTA013 was manufactured at a concentration of 1.2 mg/ml. This material was designated as LtPC05 and stored at – 80 °C. The material is still available at Allermed.

Lot XLtSTA014 was manufactured at a concentration of 1.2 mg/mL as a back-up to Lot XLtSTA012. This lot was used in cohort 4 of Allermed's phase 1 safety trial. The lot also was used in the phase 2 study in which the product was diluted by study personnel at the study site to 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL and 0.8 mg/mL for use in the dose-response part of the protocol. Dilutions were made immediately prior to the administration of the product. Based on the data from the dose-response study, the 1.2 mg/mL product was diluted at the study site to 0.3 mg/mL. This dose was used to complete the specificity and sensitivity parts of the protocol. The 1.2 mg/mL lot was re- tested (after lot-release testing) for sterility, potency and protein prior to initiating the phase 2 study on 02/13/07 (Sterility: pass/pass, Potency: 12 mm/ 11 mm, Protein: 1.15/1.08 mg/mL). Post-study stability tests were not performed on these materials, since the study was of short duration (4 months) and records at the study site showed that the product had been stored at 2-8 °C during the course of the study.

Lot XLtSTA015 was placebo/diluent that was used for Allermed's phase 2 trial; designated as XLt15-06 in the protocol.

Lot XLtSTA016 was manufactured at a protein concentration of 0.5 mg/mL and used in the validation of the guinea pig potency assay.

Lot XLtSTA017 was manufactured at a protein concentration of 0.5 mg/mL for use in Allermed's phase 2B clinical trial. The lot was also used in the validation of the guinea pig potency assay.

Lot XLtSTA018 was manufactured at a protein concentration of 0.3 mg/mL for use in Allermed's phase 2B clinical trial.

Lot XLtSTA019 was manufactured at a protein concentration of 0.15 mg/mL for use in Allermed's phase 2B clinical trial.

Comment:

Lot release results for XLtSTA017, XLtSTA018 and XLtSTA019 were used as the pre-study values for sterility, potency and protein content. The post study values were as follows: Sterility: all lots (pass); Potency: Lot XLtSTA017 (9mm), XLtSTA018 (8mm), XLtSTA019 (8mm), and Protein: XLtSTA017 (0.57 mg/mL) XLtSTA018 (0.31 mg/mL) XLtSTA019 (0.16 mg/mL).

XLtSTA020 was not formulated. Portion of the source material was accidentally spilled which resulted in a low protein value.

XLtSTA021 was used for Bioburden validation studies.

XLtSTA022 and XLtSTA023 were combined to make an internal reference standard at a protein concentration of 0.5 mg/mL. The material is labeled LtLRS01 and is stored at -80 °C.

XLtSTA024 and XLtSTA025 were combined to make a second internal reference standard at a protein concentration of 0.5 mg/mL. The material is labeled LtLRS02 and is stored at 1-5 C and -80 °C.

Comment:

Beginning with lots XLtSTA022 and XLtSTA023 two lots were combined to make a single bulk product. This procedure was introduced in our manufacturing process to increase the amount of product available to be filled in final containers. Bulk and final container testing are performed on the combined material.

XLtSTA026 and XLtSTA027 were combined to make Lot LtSTA01 containing 0.5 mg/mL of Ninhydrin protein. This lot has been placed in a long-term stability protocol.

XLtSTA028 and XLtSTA029 were combined to make Lot LtSTA02 containing 0.5 mg/mL of Ninhydrin protein. This lot has been placed in a long-term stability protocol.

XLtSTA030 and XLtSTA031 are in process and will be combined to make a third lot containing 0.5 mg/mL of Ninhydrin protein for long-term stability testing.

No other lots have been manufactured during the development of LtSTA

FDA COMMENT

8. *Because of the clinical study observations, as well as the need to demonstrate consistency of manufacture by appropriate laboratory and clinical testing, we reiterate the need to perform further physicochemical characterization of the bulk as well as final product. It is likely that development of additional lot release tests will be needed, as indicated by*

the results of product characterization. We recommend that at a minimum, these efforts include the following:

8.a. Determination of the identities of at least some of the major protein components of the bulk product, and examination of the physical state of the major components in the final product.

Based on the protein bands present in SDS-Page gels stained with Coomassie Blue, the proteins identified at 8 kDa, 20 kDa, 30 kDa, 56-58 kDa and 67 kDa were analyzed by NanoLC-ESI-MS/MS peptide sequencing technology. This work was performed by ProtTech, Inc, 2550 Boulevard of the Generals, suite 120, Norristown, PA 19403-3679 (610)635-0605. The results of their work are reported in Attachment 2. The report provides a list of the proteins identified in each band and a list of the peptides sequenced in each protein. The proteins in the 8 kDa band appear to be ubiquitin which is consistent with the fact that several proteases were present in the sample. The 56-58 kDa bands appear to contain several proteins of similar molecular weight. Using *L. major* as a reference, the compounds identified in the 20 kDa, 30 kDa, 56-58 kDa were as follows: 20 kDa (threonine peptidase and iron superoxide dismutase), 30 kDa (aldolase and cathepsn L-like protease), 56-58 kDa (metalloproteases, trypanothione reductase and dihydrolipoamide dehydrogenase). This information is summarized in the table shown in Attachment 4 with a comparison of similarities with *Leishmania* species, *Trypanosoma* and humans.

FDA COMMENT

8.b. Determination of the relative quantities of the major protein components of the bulk and final products, e.g., using scanning densitometry.

The relative quantities of the 8 kDa, 20 kDa, 30 kDa, 58 kDa and 67 KDa components have been determined by scanning densitometry. Six bands were detected in gels stained with Coomassie Blue. Six lots of drug substance and two lots of drug product were evaluated. The gel image revealed conspicuous bands at 8 kDa and 56-58 kDa in both the drug substance and drug product. Bands at 20 kDa and 30 kDa were less conspicuous in both the drug substance and drug product, and a band at 67 kDa was only visible in two lots of drug substance which had been recently manufactured relative to the other four lots of drug substance. The 67 kDa band was not visible in the drug product. In the drug substance the 56 and 58 kDa bands accounted for 60-70% of the protein in each lot; these bands were detected as a single band in the drug product accounting for 40 % of the protein in the samples. Differences identified by the densitometer in the intensity and relative percentages of each band measured in the drug substance and drug product are believed to be due to the concentration of protein present. The drug substance contained 3-5 mg/mL, whereas the drug product contained 1.5 mg/mL (note: it was necessary to concentrate the 0.5 mg/mL drug product 3X in order to visualize the bands with Coomassie Blue stain). With only one band detected at the 56-58 kDa location in the more dilute drug product, the relative percentage of each band in the drug product changed, which is

believed to account for the increase in the percentage of material at 8 kDa as shown in Attachment 2.

FDA COMMENT

8.c. Establishment of specifications for the relative quantities of the major protein components of the final product, i.e., range of relative proportions of protein bands designated as 'major '.

It is not possible to establish specifications for the relative quantities of the major protein components in the final product, due to the faintness of the protein bands when stained with Coomassie Blue. For this reason, we intend to establish specifications for the drug substance which can be stained with Coomassie Blue and analyzed by scanning densitometry. Based on the analysis of six lots of drug substance, the ranges for the major proteins in the drug substance are as follows: 8 kDa (10.1 – 31%), 20 kDa (3.9-7.8%), 30 kDa (3.4-9.2%), 56 kDa (28.9-40.1%), 58 kDa (31.3-40.1%). A range for the 67 kDa band will not be established, because of the transient nature of the band. See Attachment 2. Table 3, *Quantitation of SDS-Page gel Densitometer.*

The values reported for two lots of drug product (LtSTA01 and LtSTA02) in the table were obtained by concentrating lot samples to 1.0 mg /mL to 1.5 mg/mL in order to detect the bands that were present by scanning densitometry using Coomassie Blue Stain.

Final product will be stained using silver nitrate to verify the bands observed in the drug substance are present in the final product. At the drug product concentration of 0.5 mg/mL, the 56 kDa and 58 kDa appear as one band on sliver stained gels. This was also observed using Coomassie Blue Stain with product containing 1.5 mg/mL as shown in Table 3. Final product must contain the 8 kDa, 20 kDa, 30 kDa, and 56 kDa bands by silver stain to be acceptable. Other bands that are present in silver stained gels will not be monitored

FDA COMMENT

8.d. Determination of the quantities of carbohydrate and lipid components, and of carbohydrate and lipid modifications of protein components. These results may indicate the need for identifying individual components.

Evaluating the presence of glycoproteins and lipoproteins in the crude lysate has been accomplished by specific staining procedures. However, in-depth studies of proteins modified with these components are very complex and, in our opinion, go beyond the scope of this project. We have tested LtSTA with gel-based glycoprotein and lipoprotein assays and have not found these compounds to be present in LtSTA. The methods used in these studies and the results of the work are reported in Attachment 3.

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8.e. Comparison of the protein sequences of the major components to publicly available genomic and EST sequences of humans, other Leishmania species, and other potentially cross-reacting species.

The protein sequences of the 67 kDa, 58 kDa and 30 kDa, 20 kDa and 8 kDa components are reported in Attachment 4. This information has been compared with the publically available information regarding the genomic and EST sequences of other *Leishmania* species, closely related parasites and humans. In as much as the *L. tropica* genome has not been completely assembled, we have used the genome of *L. major*, a member of the *L. tropica* complex, as a reference for the proteins that we have sequenced in LtSTA and for a broader comparison with other genomes. As expected, when comparing two highly diverged genomes, the human genome with 3 billion bases and *L major* with 30 million bases, the two genomes are quite different. Comparison on the protein level is somewhat more informative. The protein sequences identified in the table shown in Attachment 4 were compared with the protein sequences of the human RefSeq database to see if any identified proteins had an abnormally high similarity to human protein sequences, which might suggest the possibility of cross reactivity with host proteins. None of the proteins that were identified in LtSTA had a high similarity to known human proteins. Some of the identified proteins had conserved proteases or redox domains, but these were highly diverged from the human homologues. No protein that was found in LtSTA was more similar to known human protein sequences than to a panel of highly diverged species, including Thale Cress (*Arabidopsis thaliana*), the nematode (*Caenorhabditis elegans*), pepper (*Capsicum annuum*), rice (*Oryza sativa*) and yeast (*Schizosaccharomyces japonicas*). In addition, none of the LtSTA proteins that were identified had a significantly greater similarity to human proteins than the putative dominant products in the PPD-S2 Tuberculin FDA standard. Regarding other *Leishmania* species and closely related species, the proteins identified in LtSTA at 20 kDa, 30 kDa and 56-58 kDa have a high degree of similarity to proteins of *L. braziliensis, L. infantum, L. mexicana* and to a lesser degree in *Trypanosoma cruzi* and *T. brucei*.

FDA COMMENT

9. We note that prolonged heat treatment (which might reasonably be expected to adversely affect potency) apparently did not change the outcome in your current potency assay. You state that the potency assay is under revision and that you plan to report a relative potency value. We encourage this approach, but also advise that further development of the proposed potency assay is required to ensure that the resulting format reliably distinguishes between potent, sub-potent, and suprapotent lots. As your experience with the revised assay continues, a range of error for the relative potency value should be established.

The potency test for LtSTA has been validated. The limits of the assay are 34.0 to 61.8 mcg/0.1 mL for LtSTA labeled to contain 50 mcg/0.1 mL of Ninhydrin protein. The Ninhydrin

protein assay also has been validated. The risk of producing subpotent or superpotent lots of LtSTA is low for the following reasons:

1. The Ninhydrin protein concentration (NPC) of a manufactured lot is compared with the known NPC of an internal reference standard with appropriate controls.

2. The relative potency assay is based on the side-by-side comparison of the induration response to a manufactured lot with the induration response to an internal reference standard with a Ninhydrin protein concentration of 50 mcg/0.1mL. The variability of the test is minimized, if not eliminated, when the test is performed on the same animal in parallel fashion.

References:

1. Jose et al. Evaluation of the sensitizing power of the reaction of Monetnegro. J. Brazilian Soc. Trop. Med. 2001.

2. Klein, et al. Proteophosphoglycans of *Leishmania mexicana*. Biochemical Society, 1999.

3. Soteriadou, et al. Identification of monomeric and oligomeric forms of a major *Leishmania infantum* antigen using monoclonal antibodies. Infection and Immunity, 1988.

4. Khabiri, et al. *Leishmania major*: Common antigen responsible for induction of delayed-type hypersensitivity response in guinea pigs. Parasitol . Res., 2007.

5. Beyrodt, et al. Characterization of an antigen from *Leishmania amoazonensis* amastigotes able to elicit protective responses in a murine model. Infection and Immunity, 1997.

Attachment 1

ProtTech, Inc.

REPORT: QUANTITATION OF PROTEIN BANDS WITH GEL DENSITOMETRY OF LOTS XLTSTA031 (NO PHENOL), XLTSTA031 (WITH 0.4% PHENOL)

DOCUMENT #: PTECH-110809J

INVESTIGATOR: Dr. Stewart Nielsen

AFFILIATION: AllerMed Laboratories, Inc.

REPORT DELIVERY DATE: August 9, 2011

Report Generated by: Drake X. Zhang, Ph.D.

I. GENERAL DESCRIPTION OF THE SERVICE

Protein bands were quantified with gel densitometry for sample lanes: XLtSTA031 (no phenol) and XLtSTA031 (0.4% phenol)

II. SUMMARY OF RESULTS

1, There are five bands detected for each sample lane.

2. List of quantitation results.

Band	MolWt(kD)	XLtSTA031 No Phenol	XLtSTA031 0.4% Phenol
1	67	15.7%	16.3%
2	56	38.0%	37.7%
3	30	19.0%	17.7%
4	20	12.0%	13.0%
5	8	15.3%	15.3%

III. MATERIAL AND METHODS

Material:

SDS-PAGE gel was provided by AllerMed stained with Coomassie blue with 10-20% Tris-HCL Precast Gel (Bio-Rad, Hercules, CA). (Information based on PTECH110715J)

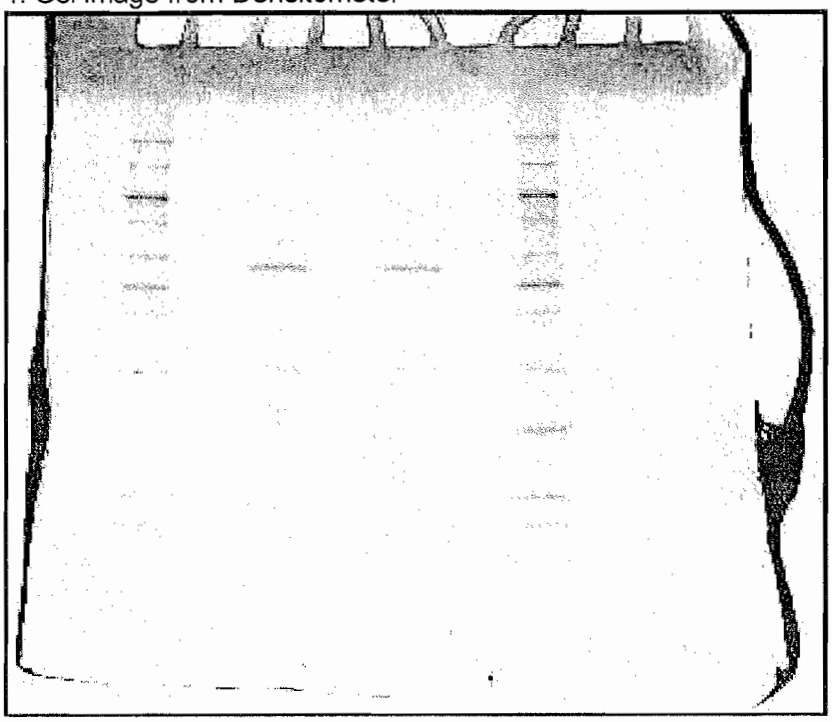
Methods:

a). Quantitative densitometry

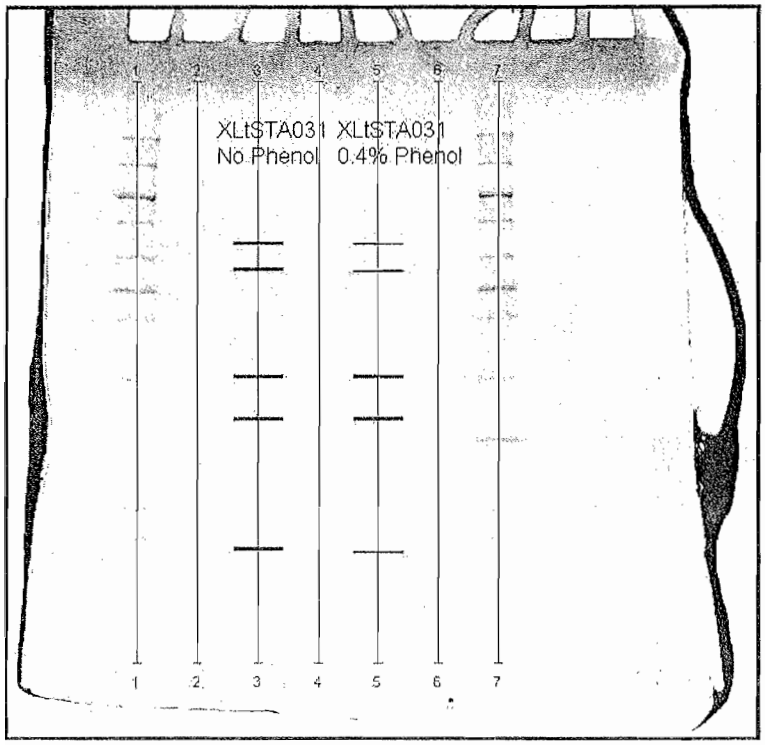
GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA) was used to scan the gels. images with transmissive mode. The resolution used is X x Y 42.3 x 42.3 micros. The images were processed with Quantity One version 4.6.8 from Bio-Rad, a software package to quantify images.

IV. RESULTS AND DISCUSSION

1. Gel Image from Densitometer



2. Gel Image from Densitometer with Labels



3. Quantitation of SDS-PAGE Gel with Densitometer

Band	MolWt(kD)	XLtSTA031 No Phenol		XLtSTA031 0.4% Phenol	
		Relative Qty	% Qty	Relative Qty	% Qty
1	67	3.4	15.7%	3.5	16.3%
2	56	8.2	38.0%	8.1	37.7%
3	30	4.1	19.0%	3.8	17.7%
4	20	2.6	12.0%	2.8	13.0%
5	8	3.3	15.3%	3.3	15.3%

V. REFERENCE

1. PT-SOP-0905 Quantitative densitometry with GS-800 Calibrated Densitometer and Quantity One software.
2. ProtTech Inc. Notebook PTLN-09331: 029.

VI. REVISION HISTORY

Version No.	Change Date.	Sections	Description of Revision
1.0	August 9, 2011	All	New document

Detail Report by Lane
PTLAB0006 2011-08-09 13hr 13min
August 9, 2011

Lane 3 Name: #3

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
None	--	10.0	5.8	0.0	4.0	1.0	5

Band #	Band Type #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty
3 - 1		0.277	0.03	0.035		3.4
3 - 2		0.323	0.11	0.083		8.2
3 - 3		0.508	0.03	0.041		4.1
3 - 4		0.579	0.02	0.026		2.6
3 - 5		0.803	0.03	0.033		3.3

Lane 5 Name: #5

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
None	--	10.0	5.8	0.0	4.0	1.0	5

Band #	Band Type #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty
5 - 1		0.279	0.03	0.033		3.5
5 - 2		0.326	0.09	0.078		8.1
5 - 3		0.508	0.03	0.036		3.8
5 - 4		0.579	0.02	0.027		2.8
5 - 5		0.808	0.03	0.032		3.3

Molecular Weight Calculation Method: Point to Point
= Known x Extrapolated

Protein Analysis Report

I. General Description of the Service

Project Name: Identification of proteins from SDS-PAGE gel bands

Sample Name: XLTSTA031---67Kd-PHENOL, 67KD-NO PHENOL, 56Kd-PHENOL, 56KD-NO PHENOL, 30Kd-PHENOL, 30KD-NO PHENOL, 20Kd-PHENOL, 20KD-NO PHENOL, 8Kd-PHENOL, 8KD-NO PHENOL

Confirmation Number: n/a

Total Number of Samples: 10

Investigator Name: Dr. Robert Bottomley

Affiliation: Allermed Laboratories

Report Delivery Date: August 22, 2011

II. Experimental

Proteomic Analysis

The protein identification work was carried out at ProtTech, Inc. by using the NanoLC-ESI-MS/MS peptide sequencing technology. In brief, each protein gel band was destained, with disulfide linkage reduced by DTT and all cysteine residues alkylated by iodoacetamid. The sample is cleaned by washing with water, and digested in-gel with sequencing grade modified trypsin (Promega) in a digestion buffer of 100 mM ammonium bicarbonate pH8.5. The peptides from a digestion were extracted out by a acetonitrile/water mixture solution, completely dried down in a vacuum (SpeedVac), re-dissolved in a sample solution of 2% acetonitrile 97.5% water, 0.5% formic acid. The dissolved peptide samples was then injected in to a high pressure liquid chromatography (HPLC) system (Beckman) with a 75 micrometer inner diameter reverse phase C18 column. HPLC Solvent A was 97.5% water, 2% acetonitrile, 0.5% formic acid. HPLC Solvent B was 9.5% water, 90% acetonitrile, 0.5% formic acid. The gradation time was 60 minutes from 2% Solvent B to 90% solvent B, plus 20 minutes for sample loading and 20 minutes for column washing. The column flow rate is around 800 nanoliter per minute. The HPLC system was on-line coupled with an ion trap mass spectrometer ((Thermo) in a way a sample eluted from HPLC column was ionized by a electrospray ionization (ESI) process and enter into the mass spectrometry. The mass spectrometer was set at data-dependent mode to acquire MS/MS data via a low energy collision induced dissociation (CID) process. The mass spectrometric data acquired were used to search the most recent non-redundant protein database with ProtTech's proprietary software suite. The output from the database search was manually validated before reporting. More experimental details can be found at www.ProtTech.com.

Materials

Endoproteinase trypsin (modified, sequencing grade) was obtained from Promega (Madison, WI). All other chemicals used in proteolytic digestion and HPLC were obtained from Sigma (St. Louis, MO). The Quadrupole ion trap mass spectrometer used in the proteomic analysis was manufactured by Thermo (Palo Alto, CA). The non-redundant protein database was downloaded from NCBI's GenBank.

III. A List of Proteins Identified in Each Sample

File Name: LC-MS/MS file name of each sample, in the format of (User Name)_(Sample Name)_Date
Hits: Numbering of all proteins identified in each sample, listed according to relative abundances.
Protein Mass: The calculated molecular weight of each identified protein based on its amino acid sequence present in the current NR database.
No. of Peptide: The number of peptides sequenced by LC-MS/MS in each identified protein.
Sequencing Header: The header of a identified protein present in NR database. It is limited to 300 characters.

BOTTOMLEYR_XLTSTA031-67Kd-PHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	76287.43	7	>gi|146077005|ref|XP_001463059.1| dipeptidyl-peptidase III; metallo-peptidase, Clan M-, Family M49 [Leishmania infantum JPCM5]ı gi|134067141|emb|CAM65406.1| dipeptidyl-peptidase III, putative; metallo-peptidase, Clan M-, Family M49 [Leishmania infantu	146077005
2	58137.39	6	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin]ı gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin	157865648
3	81041.7	4	>gi|157873023|ref|XP_001685029.1| cysteine peptidase, Clan CA, family C2; cytoskeleton-associated protein CAP5.5 [Leishmania majorı gi|68128100|emb|CAJ08231.1| cysteine peptidase, Clan CA, family C2, putative; cytoskeleton-associated protein CAP5.5, p	157873023
BOTTOMLEYR_XLTSTA031-67Kd-NOPHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	81041.7	5	>gi|157873023|ref|XP_001685029.1| cysteine peptidase, Clan CA, family C2; cytoskeleton-associated protein CAP5.5 [Leishmania majorı gi|68128100|emb|CAJ08231.1| cysteine peptidase, Clan CA, family C2, putative; cytoskeleton-associated protein CAP5.5, p	157873023
2	58137.39	4	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin]ı gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin	157865648
BOTTOMLEYR_XLTSTA031-56Kd-PHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number

1	51212.95	13	>gi|146097055|ref|XP_001468025.1| dihydrolipoamide dehydrogenase [Leishmania infantum JPCM5]ı gi|44804791|gb|AAS47708.1| dihydrolipoamide dehydrogenase [Leishmania major]ı gi|134072391|emb|CAM71099.1| dihydrolipoamide dehydrogenase, putative [Leishmani	146097055
2	57453.15	11	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain	157866094
3	53696.67	11	>gi|148283954|gb|ABQ57410.1| trypanothione reductase [Leishmania amazonensis]	148283954
4	57449.44	8	>gi|146098078|ref|XP_001468312.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]ı gi|134072679|emb|CAM71396.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]	146098078
BOTTOMLEYR_XLTSTA031-56Kd-NOPHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	57453.15	22	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain	157866094
2	51212.95	11	>gi|146097055|ref|XP_001468025.1| dihydrolipoamide dehydrogenase [Leishmania infantum JPCM5]ı gi|44804791|gb|AAS47708.1| dihydrolipoamide dehydrogenase [Leishmania major]ı gi|134072391|emb|CAM71099.1| dihydrolipoamide dehydrogenase, putative [Leishmani	146097055
3	57449.44	10	>gi|146098078|ref|XP_001468312.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]ı gi|134072679|emb|CAM71396.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]	146098078
4	53696.67	7	>gi|148283954|gb|ABQ57410.1| trypanothione reductase [Leishmania amazonensis]	148283954
BOTTOMLEYR_XLTSTA031-30Kd-PHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	30614.24	8	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]	157870820
2	30140.63	3	>gi|146079644|ref|XP_001463816.1| NGG1 interacting factor 3-like protein [Leishmania infantum JPCM5]ı gi|134067904|emb|CAM66185.1| NGG1 interacting factor 3-like protein [Leishmania infantum]	146079644
BOTTOMLEYR_XLTSTA031-30Kd-NOPHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	30614.24	9	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]	157870820
2	30140.63	1	>gi|146079644|ref|XP_001463816.1| NGG1 interacting factor 3-like protein [Leishmania infantum JPCM5]ı gi|134067904|emb|CAM66185.1| NGG1 interacting factor 3-like protein [Leishmania infantum]	146079644
BOTTOMLEYR_XLTSTA031-20Kd-PHENOL_081311				

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	24223.67	5	>gi|146103656|ref|XP_001469616.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like,threonine peptidase, Clan T(1), family T1B [Leishmania infantum]ı gi|25956176|emb|CAC82584.1| HslVU complex proteolytic subunit [Leishmani	146103656
2	51938.33	2	>gi|157875030|ref|XP_001685922.1| peptidase M20/M25/M40 [Leishmania major strain Friedlin]ı gi|68128995|emb|CAJ06416.1| peptidase M20/M25/M40, putative [Leishmania major strain Friedlin]	157875030
3	23338.26	2	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]	33340141
BOTTOMLEYR_XLTSTA031-20Kd-NOPHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	24223.67	4	>gi|146103656|ref|XP_001469616.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like,threonine peptidase, Clan T(1), family T1B [Leishmania infantum]ı gi|25956176|emb|CAC82584.1| HslVU complex proteolytic subunit [Leishmani	146103656
2	23338.26	2	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]	33340141
BOTTOMLEYR_XLTSTA031-8Kd-PHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	8543.61	18	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]	9591
BOTTOMLEYR_XLTSTA031-8Kd-NOPHENOL_081311				
Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	8543.61	15	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]	9591

Note: The results from LC-MS/MS (tandem MS) are based on independent peptide sequencing. Therefore, all proteins listed here are actual proteins present in a sample instead of "potential candidates", which are often seen in a MALDI-TOF based peptide mapping. For any identified protein reported here, there should be >98% certainty if the identification is based on LC-MS/MS sequencing of one peptide, and it will have >99.9% certainty if it is based on sequencing of two or more peptides. However, if a protein has several highly homologous isoforms, it may be difficult or even impossible to determine the exact isoform due to incomplete sequence coverage.

IV. A List of Peptides Sequenced in Each Protein Identified

BOTTOMLEYR_XLTSTA031-67Kd-PHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2850	2103.07	KQYVQPHTFISGDTVEVR	>gi|146077005|ref|XP_001463059.1| dipeptidyl-peptidase III; metallo-peptidase, Clan M-, Family M49 [Leishmania infantum JPCM5]ı gi|134067141|emb|CAM65406.1| dipeptidyl-peptidase III, putative; metallo-peptidase, Clan M-, Family M49 [Leishmania infantu

2935	1393.76	APNPIVQITENAK	
3048	1347.68	AEWEGFVAVVNK	
3123	1422.74	AGLVGLEFYTPEK	
3126	1422.74	AGLVGLEFYTPEK	
3144	1895.93	DVGPTVETNIGFIESYR	
3319	1711.84	EFAGSVEGVVESFVTR	
2689	1240.64	EKGYGGIYAVGK	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin]ı gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin
2736	1110.56	SNVTFTDISK	
2737	1110.56	SNVTFTDISK	
2901	1823.94	KGYTEVMVTALPATTSR	
2943	1695.85	GYTEVMVTALPATTSR	
2946	1695.85	GYTEVMVTALPATTSR	
2980	1698.82	SLVTEGMDEEAILHR	>gi|157873023|ref|XP_001685029.1| cysteine peptidase, Clan CA, family C2; cytoskeleton-associated protein CAP5.5 [Leishmania majorı gi|68128100|emb|CAJ08231.1| cysteine peptidase, Clan CA, family C2, putative; cytoskeleton-associated protein CAP5.5, p
3171	2699.34	VFTNYIAYDSEELPSVKVENQVR	
3297	1749.86	SYDNPAEVWTSLLQK	
3301	1749.86	SYDNPAEVWTSLLQK	
BOTTOMLEYR_XLTSTA031-67Kd-NOPHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
3033	1698.82	SLVTEGMDEEAILHR	>gi|157873023|ref|XP_001685029.1| cysteine peptidase, Clan CA, family C2; cytoskeleton-associated protein CAP5.5 [Leishmania majorı gi|68128100|emb|CAJ08231.1| cysteine peptidase, Clan CA, family C2, putative; cytoskeleton-associated protein CAP5.5, p
3260	2699.34	VFTNYIAYDSEELPSVKVENQVR	
3263	2699.34	VFTNYIAYDSEELPSVKVENQVR	
3374	1749.86	SYDNPAEVWTSLLQK	
3377	1749.86	SYDNPAEVWTSLLQK	
2651	1306.58	SVEVMNTDAEGR	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin]ı gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin
2792	1110.56	SNVTFTDISK	
2795	1110.56	SNVTFTDISK	
3002	1695.85	GYTEVMVTALPATTSR	
BOTTOMLEYR_XLTSTA031-56Kd-PHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2585	1290.63	GLDGKDETLESK	>gi|146097055|ref|XP_001468025.1| dihydrolipoamide dehydrogenase [Leishmania infantum JPCM5]ı gi|44804791|gb|AAS47708.1| dihydrolipoamide dehydrogenase [Leishmania major]ı gi|134072391|emb|CAM71099.1| dihydrolipoamide dehydrogenase, putative [Leishmani
2801	1223.69	ALTDALVKHEK	
2812	1261.63	GEGSFVNPNTIK	
2816	1261.63	GEGSFVNPNTIK	

2851	1208.63	VGKFPFSANSR	
3035	2518.26	ALLHATHLYHDAHANFAQYGLR	
3070	1376.73	LGAEVTVVEFASR	
3149	1196.64	ALTGGVEYLFK	
3152	1196.64	ALTGGVEYLFK	
3319	2318.20	KTIVATGSEPTELPFLPFDEK	
3323	2318.20	KTIVATGSEPTELPFLPFDEK	
3352	2190.11	TIVATGSEPTELPFLPFDEK	
3355	2190.11	TIVATGSEPTELPFLPFDEK	
2582	1118.51	NELGEDTVDK	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
2753	1342.72	AELTTVQQANLR	
2833	1396.71	GLFATIHETGHSK	
3076	1538.80	DAGLEVVAPEAPFPK	
3151	1423.78	LTHLLSLGAWDAK	
3154	1423.78	LTHLLSLGAWDAK	
3172	2384.25	ALLDEAETAKAELTTVQQANLR	
3175	2384.25	ALLDEAETAKAELTTVQQANLR	
3176	2384.25	ALLDEAETAKAELTTVQQANLR	
3293	1436.73	SNNDFATFLPALK	
3677	2317.27	FAEVVIGHSSAFLEFLTPLLK	
2608	1150.56	NGAVQVDAYSK	>gi|148283954|gb|ABQ57410.1| trypanothione reductase [Leishmania amazonensis]
2611	1150.56	NGAVQVDAYSK	
2612	1150.56	NGAVQVDAYSK	
2623	901.49	SQALQLDK	
2626	901.49	SQALQLDK	
2653	1363.71	NKVVNGINESYK	
2900	1570.87	SQALQLDKVGVQTGK	
3037	1636.81	TSVDNIYAIGDVTNR	
3601	3021.45	SEDPNSDVLETLDTEYILIATGSWPT R	
3605	3021.45	SEDPNSDVLETLDTEYILIATGSWPT R	
3641	2502.33	VMLTPVAINEGAAFVETVFGGKPR	
2632	847.46	SLIESATK	>gi|146098078|ref|XP_001468312.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]ı gi|134072679|emb|CAM71396.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]
2638	1206.63	LTTHAHSVWR	
2723	1287.73	SLQQVKPGYIR	
2726	1287.73	SLQQVKPGYIR	
2818	756.45	DLVALAR	
2953	1362.68	ATGETLNPEYLR	
2956	1362.68	ATGETLNPEYLR	
3013	1217.64	SATALPAEFVGR	
BOTTOMLEYR_XLTSTA031-56Kd-NOPHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header

2524	1118.51	NELGEDTVDK	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
2670	1059.54	ALLDEAETAK	
2697	1342.72	AELTTVQQANLR	
2700	1342.72	AELTTVQQANLR	
2701	1342.72	AELTTVQQANLR	
2754	1396.71	GLFATIHETGHSK	
2964	1428.83	TKLSATTPLIWAK	
2976	1538.80	DAGLEVVAPEAPFPK	
2979	1538.80	DAGLEVVAPEAPFPK	
2997	1199.69	LSATTPLIWAK	
3001	1199.69	LSATTPLIWAK	
3069	1423.78	LTHLLSLGAWDAK	
3072	1423.78	LTHLLSLGAWDAK	
3180	2384.25	ALLDEAETAKAELTTVQQANLR	
3181	2384.25	ALLDEAETAKAELTTVQQANLR	
3210	1112.62	SWLPELLQK	
3213	1112.62	SWLPELLQK	
3234	1436.73	SNNDFATFLPALK	
3237	1436.73	SNNDFATFLPALK	
3330	2475.19	PLYEALFNQYESGMTLETLEK	
3615	2317.27	FAEVVIGHSSAFLEFLTPLLK	
3618	2317.27	FAEVVIGHSSAFLEFLTPLLK	
2530	1290.63	GLDGKDETLESK	>gi|146097055|ref|XP_001468025.1| dihydrolipoamide dehydrogenase [Leishmania infantum JPCM5]ı gi|44804791|gb|AAS47708.1| dihydrolipoamide dehydrogenase [Leishmania major]ı gi|134072391|emb|CAM71099.1| dihydrolipoamide dehydrogenase, putative [Leishmani
2725	1223.69	ALTDALVKHEK	
2931	2518.26	ALLHATHLYHDAHANFAQYGLR	
2967	1376.73	LGAEVTVVEFASR	
2970	1376.73	LGAEVTVVEFASR	
3063	1196.64	ALTGGVEYLFK	
3066	1196.64	ALTGGVEYLFK	
3252	2318.20	KTIVATGSEPTELPFLPFDEK	
3255	2318.20	KTIVATGSEPTELPFLPFDEK	
3282	2190.11	TIVATGSEPTELPFLPFDEK	
3285	2190.11	TIVATGSEPTELPFLPFDEK	
2571	847.46	SLIESATK	>gi|146098078|ref|XP_001468312.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]ı gi|134072679|emb|CAM71396.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]
2680	1287.73	SLQQVKPGYIR	
2793	1362.68	ATGETLNPEYLR	
2796	1362.68	ATGETLNPEYLR	
2802	850.48	LETIFTK	
2817	1216.56	IWHFDTDAGR	
2862	1217.64	SATALPAEFVGR	
2863	1217.64	SATALPAEFVGR	
2865	1217.64	SATALPAEFVGR	
3448	2807.38	LTTNYIEDTFVQSLYGVIHESGHGK	
2554	1150.56	NGAVQVDAYSK	>gi|148283954|gb|ABQ57410.1| trypanothione reductase [Leishmania amazonensis]

2563	901.49	SQALQLDK	
2581	1121.57	VVNGINESYK	
2919	1636.81	TSVDNIYAIGDVTNR	
2922	1636.81	TSVDNIYAIGDVTNR	
3522	3021.45	SEDPNSDVLETLDTEYILIATGSWPT R	
3526	3021.45	SEDPNSDVLETLDTEYILIATGSWPT R	
BOTTOMLEYR_XLTSTA031-30Kd-PHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2889	1175.62	GLLGYVPESNK	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]
2890	1175.62	GLLGYVPESNK	
2892	1175.62	GLLGYVPESNK	
3273	2134.01	YVFPQMYFSPELQAATDK	
3276	2134.01	YVFPQMYFSPELQAATDK	
3357	1729.95	KHDVILGLFLFGTDR	
3360	1729.95	KHDVILGLFLFGTDR	
3489	2532.37	KHDVILGLFLFGTDRVGEFLEK	
3183	2085.07	LEGTTVLVSDKDRDPLAAW	>gi|146079644|ref|XP_001463816.1| NGG1 interacting factor 3-like protein [Leishmania infantum JPCM5]ı gi|134067904|emb|CAM66185.1| NGG1 interacting factor 3-like protein [Leishmania infantum]
3186	2085.07	LEGTTVLVSDKDRDPLAAW	
3234	1666.92	NVEVILAYHPPLFR	
BOTTOMLEYR_XLTSTA031-30Kd-NOPHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2953	1175.62	GLLGYVPESNK	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]
2956	1175.62	GLLGYVPESNK	
3307	2134.01	YVFPQMYFSPELQAATDK	
3310	2134.01	YVFPQMYFSPELQAATDK	
3403	1729.95	KHDVILGLFLFGTDR	
3406	1729.95	KHDVILGLFLFGTDR	
3469	1601.86	HDVILGLFLFGTDR	
3473	1601.86	HDVILGLFLFGTDR	
3535	2532.37	KHDVILGLFLFGTDRVGEFLEK	
3191	2085.07	LEGTTVLVSDKDRDPLAAW	>gi|146079644|ref|XP_001463816.1| NGG1 interacting factor 3-like protein [Leishmania infantum JPCM5]ı gi|134067904|emb|CAM66185.1| NGG1 interacting factor 3-like protein [Leishmania infantum]
BOTTOMLEYR_XLTSTA031-20Kd-PHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header

2835	1217.60	LNDFPEQLSR	>gi|146103656|ref|XP_001469616.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like,threonine peptidase, Clan T(1), family T1B [Leishmania infantum]ı gi|25956176|emb|CAC82584.1| HslVU complex proteolytic subunit [Leishmani
2838	1217.60	LNDFPEQLSR	
2880	1335.63	ALIDVDGYDAER	
2883	1335.63	ALIDVDGYDAER	
2913	1701.87	LENKLNDFPEQLSR	
3259	1614.89	TPFLLVEIAGTEPTK	>gi|157875030|ref|XP_001685922.1| peptidase M20/M25/M40 [Leishmania major strain Friedlin]ı gi|68128995|emb|CAJ06416.1| peptidase M20/M25/M40, putative [Leishmania major strain Friedlin]
3420	2126.06	GGADDGYALFSAITSVSVLQR	
2473	1153.55	EQVTFHHEK	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]
2664	1332.69	LTAAAESNSALASK	
BOTTOMLEYR_XLTSTA031-20Kd-NOPHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2882	1217.60	LNDFPEQLSR	>gi|146103656|ref|XP_001469616.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like,threonine peptidase, Clan T(1), family T1B [Leishmania infantum]ı gi|25956176|emb|CAC82584.1| HslVU complex proteolytic subunit [Leishmani
2885	1217.60	LNDFPEQLSR	
2951	1335.63	ALIDVDGYDAER	
2954	1335.63	ALIDVDGYDAER	
2525	1153.55	EQVTFHHEK	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]
2528	1153.55	EQVTFHHEK	
BOTTOMLEYR_XLTSTA031-8Kd-PHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2506	1522.77	IQDKEGIPPDQQR	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]
2509	1522.77	IQDKEGIPPDQQR	
2536	1522.77	IQDKEGIPPDQQR	
2539	1522.77	IQDKEGIPPDQQR	
2563	1522.77	IQDKEGIPPDQQR	
2566	1522.77	IQDKEGIPPDQQR	
2605	1522.77	IQDKEGIPPDQQR	
2608	1522.77	IQDKEGIPPDQQR	
2615	1080.54	TLSDYNIQK	
2830	1359.75	LIFAGKQLEEGR	
2836	1066.61	ESTLHLVLR	
2839	1066.61	ESTLHLVLR	
2950	1756.91	TIALEVEPSDTIENVK	
2953	1756.91	TIALEVEPSDTIENVK	
2954	1756.91	TIALEVEPSDTIENVK	
2984	1756.91	TIALEVEPSDTIENVK	
3010	1756.91	TIALEVEPSDTIENVK	
3016	1756.91	TIALEVEPSDTIENVK	
BOTTOMLEYR_XLTSTA031-8Kd-NOPHENOL_081311			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header

2545	1522.77	IQDKEGIPPDQQR	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]
2548	1522.77	IQDKEGIPPDQQR	
2572	1522.77	IQDKEGIPPDQQR	
2575	1522.77	IQDKEGIPPDQQR	
2602	1522.77	IQDKEGIPPDQQR	
2605	1522.77	IQDKEGIPPDQQR	
2629	1522.77	IQDKEGIPPDQQR	
2632	1522.77	IQDKEGIPPDQQR	
2656	1522.77	IQDKEGIPPDQQR	
2665	1522.77	IQDKEGIPPDQQR	
2669	1522.77	IQDKEGIPPDQQR	
3070	1756.91	TIALEVEPSDTIENVK	
3073	1756.91	TIALEVEPSDTIENVK	
3097	1756.91	TIALEVEPSDTIENVK	
3100	1756.91	TIALEVEPSDTIENVK	

Scan No: The scan number of an identified peptide in a LC-MS/MS file. If a peptide has been sequenced more than once, it will have more than one scan numbers.
Peptide Mass: The calculated molecular weight of each peptide based on its amino acid sequence.
Peptide Sequence: The sequence of each peptide identified
Sequence Header: The header of a identified protein present in NR database. It is limited to 300 characters.

For gel samples, since the proteins from same gel bands often have similar mass, the number of peptides sequenced by LC-MS/MS from each protein can be used as an indication for relative abundance of proteins in a sample. For this reason, one peptide sequence may be present several times in the table below if it has more than one LC-MS/MS scans.

BOTTOMLEYR_XLTSTA031-67Kd-PHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	76287.43	7	>gi|146077005|ref|XP_001463059.1| dipeptidyl-peptidase III; metallo-peptidase, Clan M-, Family M49 [Leishmania infantum JPCM5] gi|134067141|emb|CAM65406.1| dipeptidyl-peptidase III, putative; metallo-peptidase, Clan M-, Family M49 [Leishmania infantu	146077005
2	58137.39	6	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin] gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin	157865648
3	81041.7	4	>gi|157873023|ref|XP_001685029.1| cysteine peptidase, Clan CA, family C2; cytoskeleton-associated protein CAP5.5 [Leishmania major gi|68128100|emb|CAJ08231.1| cysteine peptidase, Clan CA, family C2, putative; cytoskeleton-associated protein CAP5.5, p	157873023

BOTTOMLEYR_XLTSTA031-67Kd-NOPHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	81041.7	5	>gi|157873023|ref|XP_001685029.1| cysteine peptidase, Clan CA, family C2; cytoskeleton-associated protein CAP5.5 [Leishmania major gi|68128100|emb|CAJ08231.1| cysteine peptidase, Clan CA, family C2, putative; cytoskeleton-associated protein CAP5.5, p	157873023
2	58137.39	4	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin] gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin	157865648

BOTTOMLEYR_XLTSTA031-56Kd-PHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	51212.95	13	>gi|146097055|ref|XP_001468025.1| dihydrolipoamide dehydrogenase [Leishmania infantum JPCM5] gi|44804791|gb|AAS47708.1| dihydrolipoamide dehydrogenase [Leishmania major] gi|134072391|emb|CAM71099.1| dihydrolipoamide dehydrogenase, putative [Leishmani	146097055
2	57453.15	11	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin] gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain	157866094
3	53696.67	11	>gi|148283954|gb|ABQ57410.1| trypanothione reductase [Leishmania amazonensis]	148283954
4	57449.44	8	>gi|146098078|ref|XP_001468312.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum] gi|134072679|emb|CAM71396.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]	146098078

BOTTOMLEYR_XLTSTA031-56Kd-NOPHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	57453.15	22	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin] gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain	157866094
2	51212.95	11	>gi|146097055|ref|XP_001468025.1| dihydrolipoamide dehydrogenase [Leishmania infantum JPCM5] gi|44804791|gb|AAS47708.1| dihydrolipoamide dehydrogenase [Leishmania major] gi|134072391|emb|CAM71099.1| dihydrolipoamide dehydrogenase, putative [Leishmani	146097055
3	57449.44	10	>gi|146098078|ref|XP_001468312.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum] gi|134072679|emb|CAM71396.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania infantum]	146098078
4	53696.67	7	>gi|148283954|gb|ABQ57410.1| trypanothione reductase [Leishmania amazonensis]	148283954

BOTTOMLEYR_XLTSTA031-30Kd-PHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	30614.24	8	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin] gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]	157870820

			>gi|146079644|ref|XP_001463816.1| NGG1 interacting factor 3-like protein [Leishmania infantum JPCM5] gi|134067904|emb|CAM66185.1| NGG1	
2	30140.63	3	interacting factor 3-like protein [Leishmania infantum]	146079644

BOTTOMLEYR_XLTSTA031-30Kd-NOPHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
			>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin] gi|68127027|emb|CAJ05502.1| hypothetical protein,	
1	30614.24	9	conserved [Leishmania major strain Friedlin]	157870820
			>gi|146079644|ref|XP_001463816.1| NGG1 interacting factor 3-like protein [Leishmania infantum JPCM5] gi|134067904|emb|CAM66185.1| NGG1	
2	30140.63	1	interacting factor 3-like protein [Leishmania infantum]	146079644

BOTTOMLEYR_XLTSTA031-20Kd-PHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
			>gi|146103656|ref|XP_001469616.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like,threonine peptidase, Clan T(1), family T1B [Leishmania infantum] gi|25956176|emb|CAC82584.1| HslVU complex	
1	24223.67	5	proteolytic subunit [Leishmani	146103656
			>gi|157875030|ref|XP_001685922.1| peptidase M20/M25/M40 [Leishmania major strain Friedlin] gi|68128995|emb|CAJ06416.1| peptidase	
2	51938.33	2	M20/M25/M40, putative [Leishmania major strain Friedlin]	157875030
			>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2	
3	23338.26	2	[Leishmania major]	33340141

BOTTOMLEYR_XLTSTA031-20Kd-NOPHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
			>gi|146103656|ref|XP_001469616.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like,threonine peptidase, Clan T(1), family T1B [Leishmania infantum] gi|25956176|emb|CAC82584.1| HslVU complex	
1	24223.67	4	proteolytic subunit [Leishmani	146103656
			>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2	
2	23338.26	2	[Leishmania major]	33340141

BOTTOMLEYR_XLTSTA031-8Kd-PHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	8543.61	18	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]	9591

BOTTOMLEYR_XLTSTA031-8Kd-NOPHENOL_081311

Hits	Protein Mass	No. of Peptide	Sequence Header	gi number
1	8543.61	15	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]	9591

Relative Abundance	Score	SI	AA No	SIn*1E6
14.3%	102	9567721	679	228.89
50.2%	76	26662687	538	805.02
35.5%	59	25331841	723	569.13

Relative Abundance	Score	SI	AA No	SIn*1E6
71.8%	72	54476677	723	1070.08
28.2%	42	15936638	538	420.69

Relative Abundance	Score	SI	AA No	SIn*1E6
37.9%	184	231574207	476	772.32
44.1%	176	284624874	503	898.30
12.3%	135	77784974	491	251.49
5.6%	91	35936599	499	114.33

Relative Abundance	Score	SI	AA No	SIn*1E6
54.3%	334	247882044	503	1100.33
31.9%	167	137906963	476	646.88
5.5%	110	24875838	499	111.31
8.3%	89	37207533	491	169.20

Relative Abundance	Score	SI	AA No	SIn*1E6
93.3%	128	157365434	279	3345.61

6.7%	41	11223689	279	238.62

Relative Abundance	Score	SI	AA No	SIn*1E6
95.8%	143	80077275	279	3433.40
4.2%	13	3517822	279	150.83

Relative Abundance	Score	SI	AA No	SIn*1E6
49.1%	60	11996937	220	2025.95
10.7%	40	5636067	472	443.63
40.2%	28	9283467	208	1658.17

Relative Abundance	Score	SI	AA No	SIn*1E6
71.7%	55	18840001	220	3311.93
28.3%	18	7016925	208	1304.69

Relative Abundance	Score	SI	AA No	SIn*1E6
100.0%	252	499134752	76	13157.89

Relative Abundance	Score	SI	AA No	SIn*1E6
100.0%	204	73129679	76	13157.89

Attachment 2

ProtTech, Inc.

REPORT: QUANTITATION OF PROTEIN BANDS WITH GEL DENSITOMETRY OF LOTS XLTSTA026, XLTSTA027, XLTSTA028, XLTSTA029, XLTSTA030, XLTSTA031, LTSTA01 AND LTSTA02.

DOCUMENT #: PTECH-110715J

INVESTIGATOR: Dr. Stewart Nielsen

AFFILIATION: AllerMed Laboratories, Inc.

REPORT DELIVERY DATE: July 15, 2011

Report Generated by: Drake X. Zhang, Ph.D.

I. GENERAL DESCRIPTION OF THE SERVICE

Protein bands were quantified with gel densitometry for sample lanes: XLtSTA026, XLtSTA027, XLtSTA028, XLtSTA029, XLtSTA030, XLtSTA031, LtSTA01 and LtSTA02

II. SUMMARY OF RESULTS

1, There are maximum of six bands detected. For LtSTA01 and LtSTA02, band 2 and 3 are not resolved due to low sample amount.

2. Band 1 was detected only in XLtSTA030, XLtSTA031.

III. MATERIAL AND METHODS

Material:

SDS-PAGE gel was provided by AllerMed stained with Coomassie blue with 10-20% Tris-HCL Precast Gel (Bio-Rad, Hercules, CA).

Methods:

a). Quantitative densitometry
GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA) was used to scan the gels. images with transmissive mode. The resolution used is X x Y 42.3 x 42.3 micros. The images were processed with Quantity One version 4.6.8 from Bio-Rad, a software package to quantify images.

IV. RESULTS AND DISCUSSION

1. Gel Image from Densitometer

3. Quantitation of SDS-PAGE Gel with Densitometer

	Protein	3.3 ug/ml	5.4 ug/ml	4.6 ug/ml	4.7 ug/ml	4.7 ug/ml	3.6 ug/ml	1.5 ug/ml	1.5 ug/ml
Band	MolWt(kD)	XLtSTA026	XLtSTA027	XLtSTA028	XLtSTA029	XLtSTA030	XLtSTA031	LtSTA01	LtSTA02
1	67					7.7%	6.2%		
2	58	31.3%	43.3%	36.0%	32.9%	33.4%	34.5%	38.5%	43.0%
3	56	28.9%	32.4%	40.1%	38.8%	30.7%	39.5%		
4	30	3.4%	5.0%	4.5%	5.9%	9.2%	5.8%	8.2%	11.8%
5	20	5.4%	6.7%	5.0%	7.8%	6.5%	3.9%	12.5%	9.7%
6	8	31.0%	12.6%	14.5%	14.6%	12.5%	10.1%	40.9%	35.5%

V. REFERENCE

1. PT-SOP-0905 Quantitative densitometry with GS-800 Calibrated Densitometer and Quantity One software.
2. ProtTech Inc. Notebook PTLN-09331: 028.

VI. REVISION HISTORY

Version No.	Change Date.	Sections	Description of Revision
1.0	July 15, 2011	All	New document

Figure 1. Gel Image from Densitometer

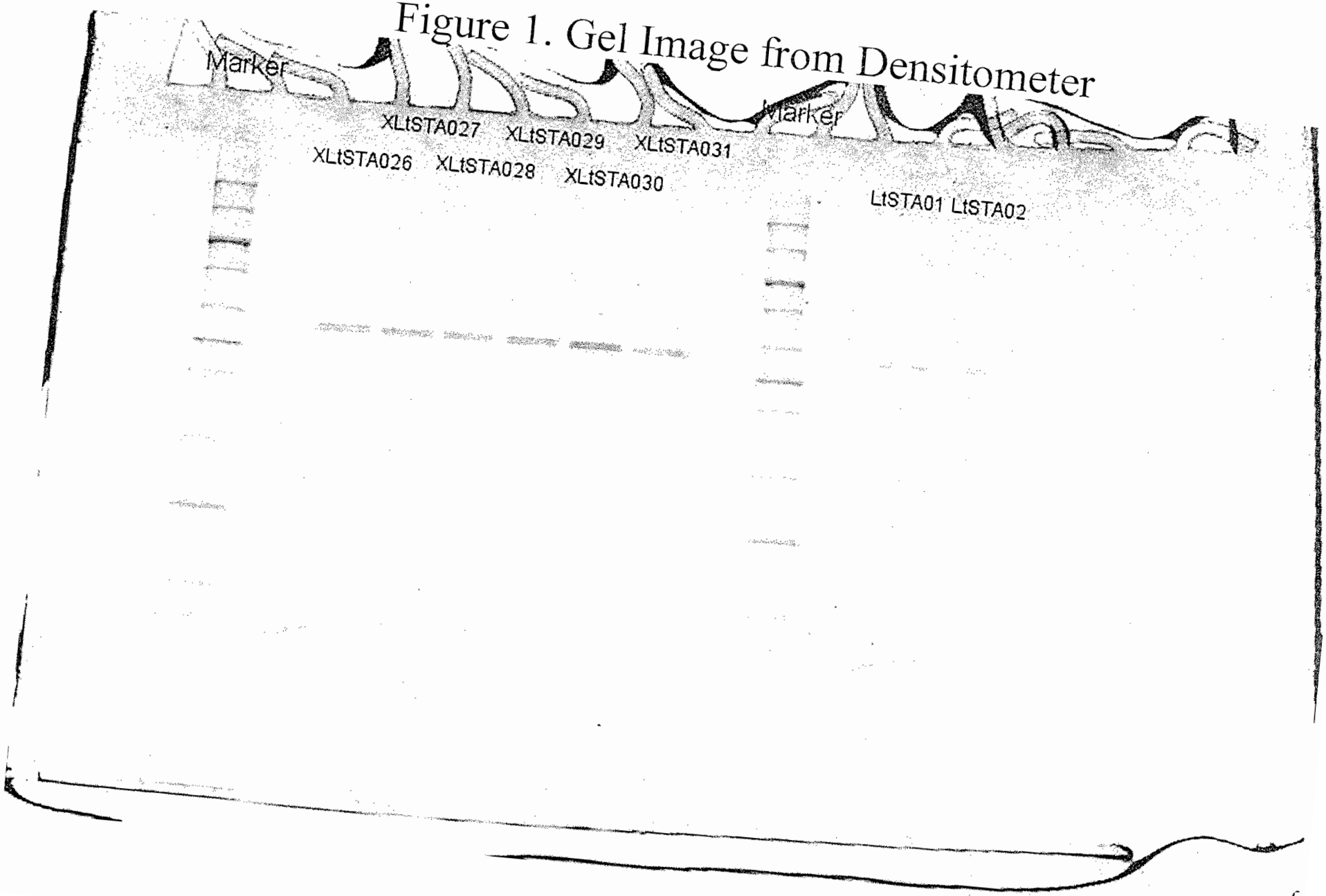
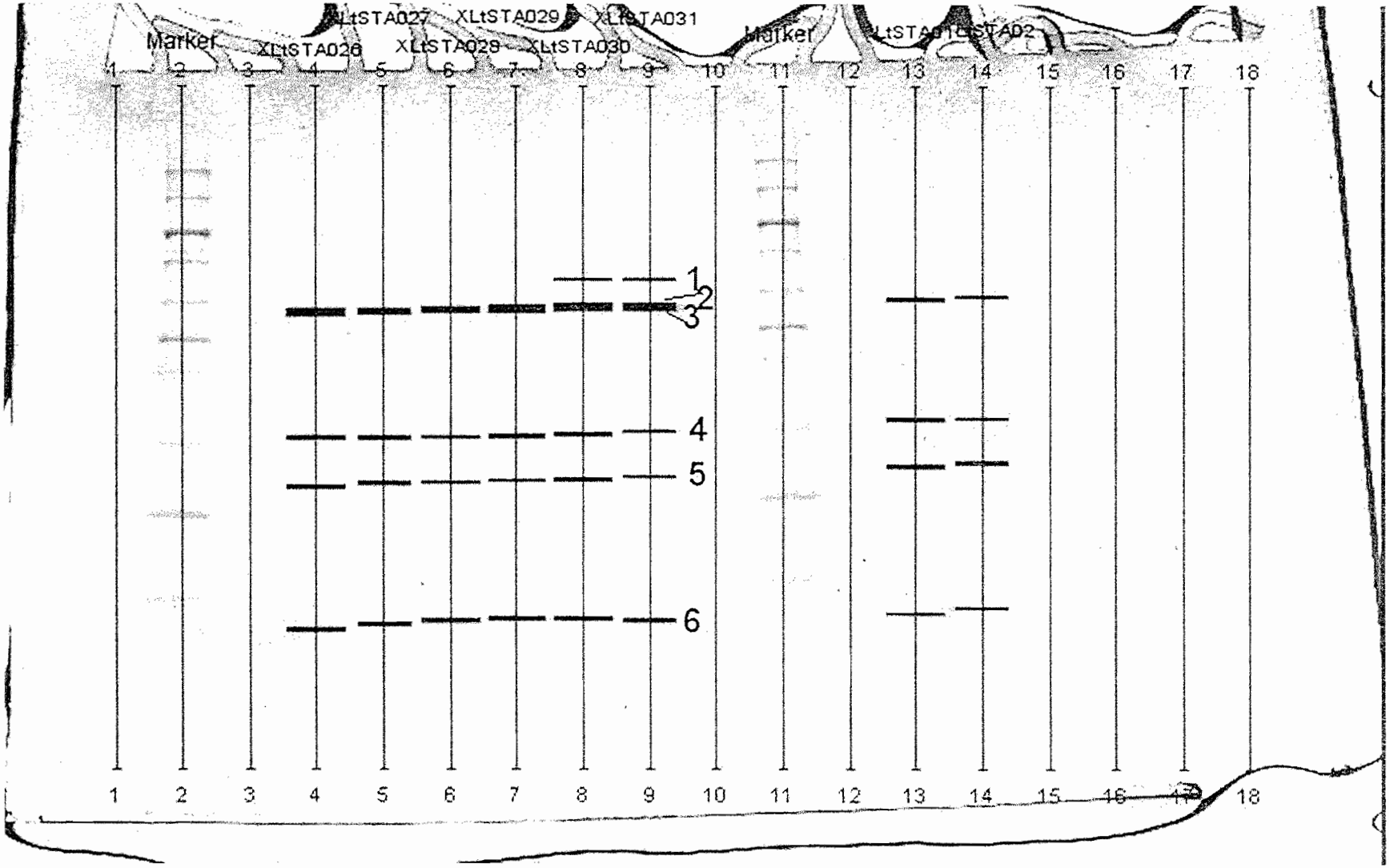


Figure 2. Gel Image from Densitometer with Labels



ProtTech Inc.

Detail Report by Lane
AllerMed_20110713_2_LABEL
July 14, 2011

Lane 4 Name: #4

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	602.4	6.5	20.0	12.6	0.3	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
4 - 5	0.330	0.07	0.038		9.2	
4 - 6	0.336	0.06	0.035		8.5	
4 - 7	0.798	0.02	0.037		9.1	
4 - 10	0.517	0.01	0.004		1.0	
4 - 11	0.589	0.01	0.006		1.6	

Lane 5 Name: #5

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	55.6	5.9	0.0	4.8	0.3	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
5 - 1	0.329	0.07	0.031		10.3	
5 - 2	0.334	0.06	0.024		7.7	
5 - 5	0.584	0.01	0.005		1.6	
5 - 6	0.789	0.03	0.009		3.0	
5 - 10	0.517	0.01	0.004		1.2	

Molecular Weight Calculation Method: Point to Point
= Known x Extrapolated

Detail Report by Lane
AllerMed_20110713_2_LABEL
July 14, 2011

Lane 6 Name: #6

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	55.6	6.5	0.0	4.8	0.3	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
6 - 1	0.326	0.06	0.025		8.7	
6 - 2	0.331	0.06	0.028		9.7	
6 - 8	0.785	0.03	0.010		3.5	
6 - 10	0.515	0.01	0.003		1.1	
6 - 11	0.582	0.01	0.003		1.2	

Lane 7 Name: #7

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	55.6	6.5	0.0	4.8	0.3	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
7 - 1	0.323	0.06	0.025		7.2	
7 - 2	0.330	0.07	0.030		8.5	
7 - 6	0.514	0.01	0.004		1.3	
7 - 7	0.580	0.02	0.006		1.7	
7 - 8	0.782	0.03	0.011		3.2	

Molecular Weight Calculation Method: Point to Point
= Known x Extrapolated

Detail Report by Lane
AllerMed_20110713_2_LABEL
July 14, 2011

Lane 8 Name: #8

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	662.6	6.5	20.0	12.6	0.4	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
8 - 4	0.322	0.13	0.061		16.0	
8 - 5	0.328	0.11	0.056		14.7	
8 - 6	0.781	0.03	0.023		6.0	
8 - 7	0.284	0.02	0.014		3.7	
8 - 9	0.510	0.02	0.017		4.4	
8 - 10	0.578	0.01	0.012		3.1	

Lane 9 Name: #9

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	55.6	5.9	0.0	4.8	0.3	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
9 - 1	0.322	0.09	0.032		8.9	
9 - 2	0.327	0.07	0.037		10.2	
9 - 4	0.284	0.02	0.006		1.6	
9 - 5	0.507	0.02	0.005		1.5	
9 - 6	0.573	0.01	0.004		1.0	
9 - 7	0.784	0.02	0.009		2.6	

Molecular Weight Calculation Method: Point to Point
= Known x Extrapolated

Detail Report by Lane
AllerMed_20110713_2_LABEL
July 14, 2011

Lane 13 Name: #13 10

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	662.6	6.5	20.0	12.6	0.4	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
13 - 3	0.313	0.03	0.024		8.0	
13 - 8	0.489	0.01	0.005		1.7	
13 - 9	0.558	0.01	0.008		2.6	
13 - 10	0.774	0.02	0.025		8.5	

Lane 14 Name: #14 11

Lane Information:

Background		Band Detection Parameters					
Method	Radius	Sensitivity	Width	Min.Dens	Filter	Shoulder	Size
Disk	100	55.6	5.9	0.0	4.8	0.3	3

Band #	Relative Front	Peak OD	Trace OD x mm	Gauss Peak OD	Relative Qty	Calibrated Qty
14 - 1	0.309	0.03	0.011		4.0	
14 - 2	0.487	0.01	0.003		1.1	
14 - 3	0.553	0.01	0.002		0.9	
14 - 4	0.766	0.03	0.009		3.3	

Molecular Weight Calculation Method: Point to Point
= Known x Extrapolated

Protein Analysis Report

I. General Description of the Service

Project Name: Identification of proteins from SDS-PAGE gel bands

Sample Name: XLtSTA028-8KD, 20KD, 30KD, 56KD, 58KD, XLtSTA30-67KD, LtSTA02-8KD, 20KD, 30KD, 58KD,

Confirmation Number: n/a

Total Number of Samples: 10

Investigator Name: Dr. Robert Bottomley

Affiliation: Allermed Laboratories

Report Delivery Date: July 29, 2011

II. Experimental

Proteomic Analysis

The protein identification work was carried out at ProtTech, Inc. by using the NanoLC-ESI-MS/MS peptide sequencing technology. In brief, each protein gel band was destained, with disulfide linkage reduced by DTT and all cysteine residues alkylated by iodoacetamid. The sample is cleaned by washing with water, and digested in-gel with sequencing grade modified trypsin (Promega) in a digestion buffer of 100 mM ammonium bicarbonate pH8.5. The peptides from a digestion were extracted out by a acetonitrile/water mixture solution, completely dried down in a vacuum (SpeedVac), re-dissolved in a sample solution of 2% acetonitrile 97.5% water, 0.5% formic acid. The dissolved peptide samples was then injected in to a high pressure liquid chromatography (HPLC) system (Beckman) with a 75 micrometer inner diameter reverse phase C18 column. HPLC Solvent A was 97.5% water, 2% acetonitrile, 0.5% formic acid. HPLC Solvent B was 9.5% water, 90% acetonitrile, 0.5% formic acid. The gradation time was 60 minutes from 2% Solvent B to 90% solvent B, plus 20 minutes for sample loading and 20 minutes for column washing. The column flow rate is around 800 nanoliter per minute. The HPLC system was on-line coupled with an ion trap mass spectrometer ((Thermo) in a way a sample eluted from HPLC column was ionized by a electrospray ionization (ESI) process and enter into the mass spectrometry. The mass spectrometer was set at data-dependent mode to acquire MS/MS data via a low energy collision induced dissociation (CID) process. The mass spectrometric data acquired were used to search the most recent non-redundant protein database with ProtTech's proprietary software suite. The output from the database search was manually validated before reporting. More experimental details can be found at www.ProtTech.com.

Materials

Endoproteinase trypsin (modified, sequencing grade) was obtained from Promega (Madison, WI). All other chemicals used in proteolytic digestion and HPLC were obtained from Sigma (St. Louis, MO). The Quadrupole ion trap mass spectrometer used in the proteomic analysis was manufactured by Thermo (Palo Alto, CA). The non-redundant protein database was downloaded from NCBI's GenBank.

III. A List of Proteins Identified in Each Sample

BOTTOMLEYR_LTSTA02-8KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	8543.61	9	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]
2	57453.15	4	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
BOTTOMLEYR_XLTSTA028-8KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	8543.61	24	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]
2	16800.53	4	>gi|157874264|ref|XP_001685619.1| nucleoside diphosphate kinase b [Leishmania major]ı gi|5852124|emb|CAB55369.1| nucleoside diphosphate kinase B [Leishmania major]ı gi|68128691|emb|CAJ08823.1| nucleoside diphosphate kinase b [Leishmania major strain Fr
BOTTOMLEYR_LTSTA02-20KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	23449.27	5	>gi|157877319|ref|XP_001686982.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like, threonine peptidase, Clan T(1), family T1B [Leishmania major]ı gi|68130057|emb|CAJ09365.1| hs1vu complex proteolytic subunit-like; hs1vu
2	23338.26	2	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]
BOTTOMLEYR_XLTSTA028-20KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	23449.27	11	>gi|157877319|ref|XP_001686982.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like, threonine peptidase, Clan T(1), family T1B [Leishmania major]ı gi|68130057|emb|CAJ09365.1| hs1vu complex proteolytic subunit-like; hs1vu
2	23338.26	2	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]
3	26374.06	1	>gi|2352425|gb|AAC38830.1| iron superoxide dismutase [Leishmania chagasi]
BOTTOMLEYR_LTSTA02-30KD_072411			
Hits	Protein	No. of	Sequence Header

	Mass	Peptide	
1	30614.24	11	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]
2	19460.04	2	>gi|87246235|gb|ABD35294.1| cysteine proteinase [Leishmania tropica]
BOTTOMLEYR_XLTSTA028-30KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	30614.24	10	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]
2	19460.04	2	>gi|87246235|gb|ABD35294.1| cysteine proteinase [Leishmania tropica]
BOTTOMLEYR_LTSTA02-58KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	57469.55	15	>gi|157875225|ref|XP_001686015.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strain Friedlin]ı gi|68129088|emb|CAJ06700.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strai
2	57453.15	9	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
3	53737.63	6	>gi|157863924|ref|XP_001687512.1| trypanothione reductase [Leishmania major]ı gi|68223723|emb|CAJ01955.1| trypanothione reductase [Leishmania major strain Friedlin]
4	51172.95	1	>gi|157874347|ref|XP_001685657.1| dihydrolipoamide dehydrogenase [Leishmania major strain Friedlin]ı gi|68128729|emb|CAJ08862.1| dihydrolipoamide dehydrogenase, putative [Leishmania major strain Friedlin]
BOTTOMLEYR_XLTSTA028-56KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	57469.55	12	>gi|157875225|ref|XP_001686015.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strain Friedlin]ı gi|68129088|emb|CAJ06700.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strai
2	51172.95	11	>gi|157874347|ref|XP_001685657.1| dihydrolipoamide dehydrogenase [Leishmania major strain Friedlin]ı gi|68128729|emb|CAJ08862.1| dihydrolipoamide dehydrogenase, putative [Leishmania major strain Friedlin]
3	57453.15	8	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
4	53737.63	7	>gi|157863924|ref|XP_001687512.1| trypanothione reductase [Leishmania major]ı gi|68223723|emb|CAJ01955.1| trypanothione reductase [Leishmania major strain Friedlin]
BOTTOMLEYR_XLTSTA028-58KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header

1	57453.15	20	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
2	53737.63	6	>gi|157863924|ref|XP_001687512.1| trypanothione reductase [Leishmania major]ı gi|68223723|emb|CAJ01955.1| trypanothione reductase [Leishmania major strain Friedlin]
BOTTOMLEYR_XLTSTA030-67KD_072411			
Hits	Protein Mass	No. of Peptide	Sequence Header
1	58137.39	6	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin]ı gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin
2	57453.15	2	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain

File Name: LC-MS/MS file name of each sample, in the format of (User Name)_(Sample Name)_Date
Hits: Numbering of all proteins identified in each sample, listed according to relative abundances.
Protein Mass: The calculated molecular weight of each identified protein based on its amino acid sequence present in the current NR database.
No. of Peptide: The number of peptides sequenced by LC-MS/MS in each identified protein.
Sequencing Header: The header of a identified protein present in NR database. It is limited to 300 characters.

Note: The results from LC-MS/MS (tandem MS) are based on independent peptide sequencing. Therefore, all proteins listed here are actual proteins present in a sample instead of "potential candidates", which are often seen in a MALDI-TOF based peptide mapping. For any identified protein reported here, there should be >98% certainty if the identification is based on LC-MS/MS sequencing of one peptide, and it will have >99.9% certainty if it is based on sequencing of two or more peptides. However, if a protein has several highly homologous isoforms, it may be difficult or even impossible to determine the exact isoform due to incomplete sequence coverage.

IV. A List of Peptides Sequenced in Each Protein Identified

BOTTOMLEYR_LTSTA02-8KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2500	1522.77	IQDKEGIPPDQQR	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]
2564	1522.77	IQDKEGIPPDQQR	
2567	1522.77	IQDKEGIPPDQQR	
2854	1756.91	TIALEVEPSDTIENVK	
2857	1756.91	TIALEVEPSDTIENVK	
2881	1756.91	TIALEVEPSDTIENVK	
2887	1756.91	TIALEVEPSDTIENVK	
2890	1756.91	TIALEVEPSDTIENVK	
2959	2841.50	QLEEGRTLSDYNIQKESTLHLVLR	

2644	1342.72	AELTTVQQANLR	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
2924	1538.80	DAGLEVVAPEAPFPK	
3470	2317.27	FAEVVIGHSSAFLEFLTPLLK	
3472	2317.27	FAEVVIGHSSAFLEFLTPLLK	
BOTTOMLEYR_XLTSTA028-8KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2439	1522.77	IQDKEGIPPDQQR	>gi|9591|emb|CAA78598.1| Ubiquitin [Leishmania major]
2442	1522.77	IQDKEGIPPDQQR	
2466	1522.77	IQDKEGIPPDQQR	
2470	1522.77	IQDKEGIPPDQQR	
2508	1522.77	IQDKEGIPPDQQR	
2521	1522.77	IQDKEGIPPDQQR	
2527	1522.77	IQDKEGIPPDQQR	
2539	1080.54	TLSDYNIQK	
2544	1080.54	TLSDYNIQK	
2547	1080.54	TLSDYNIQK	
2739	1066.61	ESTLHLVLR	
2742	1066.61	ESTLHLVLR	
2746	1359.75	LIFAGKQLEEGR	
2769	1066.61	ESTLHLVLR	
2772	1066.61	ESTLHLVLR	
2796	1066.61	ESTLHLVLR	
2799	1066.61	ESTLHLVLR	
2866	1756.91	TIALEVEPSDTIENVK	
2877	1756.91	TIALEVEPSDTIENVK	
2883	1756.91	TIALEVEPSDTIENVK	
2886	1756.91	TIALEVEPSDTIENVK	
2910	2257.20	TLTGKTIALEVEPSDTIENVK	
2916	2129.15	TLSDYNIQKESTLHLVLR	
2955	2841.50	QLEEGRTLSDYNIQKESTLHLVLR	
2652	1329.74	TFIAVKPDGVQR	>gi|157874264|ref|XP_001685619.1| nucleoside diphosphate kinase b [Leishmania major]ı gi|5852124|emb|CAB55369.1| nucleoside diphosphate kinase B [Leishmania major]ı gi|68128691|emb|CAJ08823.1| nucleoside diphosphate kinase b [Leishmania major strain Fr
2655	1329.74	TFIAVKPDGVQR	
2733	1708.91	VLLGATNPADSQPGTIR	
2736	1708.91	VLLGATNPADSQPGTIR	
BOTTOMLEYR_LTSTA02-20KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2675	1217.60	LNDFPEQLSR	>gi|157877319|ref|XP_001686982.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like, threonine peptidase, Clan T(1), family T1B [Leishmania major]ı gi|68130057|emb|CAJ09365.1| hs1vu complex proteolytic subunit-like; hs1vu
2678	1217.60	LNDFPEQLSR	
2762	1335.63	ALIDVDGYDAER	
2765	1335.63	ALIDVDGYDAER	
3185	2307.14	IATDIDVFSNSNWDVEILTR	

2459	1332.69	LTAAAESNSALASK	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]
2462	1332.69	LTAAAESNSALASK	
BOTTOMLEYR_XLTSTA028-20KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2640	1217.60	LNDFPEQLSR	>gi|157877319|ref|XP_001686982.1| hs1vu complex proteolytic subunit-like; hs1vu complex proteolytic subunit-like, threonine peptidase, Clan T(1), family T1B [Leishmania major]ı gi|68130057|emb|CAJ09365.1| hs1vu complex proteolytic subunit-like; hs1vu
2644	1217.60	LNDFPEQLSR	
2649	1217.60	LNDFPEQLSR	
2653	1217.60	LNDFPEQLSR	
2656	1217.60	LNDFPEQLSR	
2694	1335.63	ALIDVDGYDAER	
2697	1335.63	ALIDVDGYDAER	
3163	2307.14	IATDIDVFSNSNWDVEILTR	
3663	2307.14	IATDIDVFSNSNWDVEILTR	
3682	2307.14	IATDIDVFSNSNWDVEILTR	
3739	2307.14	IATDIDVFSNSNWDVEILTR	
2433	1332.69	LTAAAESNSALASK	>gi|33340141|gb|AAQ14558.1|AF312583_1 iron superoxide dismutase B2 [Leishmania major]
2436	1332.69	LTAAAESNSALASK	
2536	1581.83	HHSAYVDKLNTLGK	>gi|2352425|gb|AAC38830.1| iron superoxide dismutase [Leishmania chagasi]
BOTTOMLEYR_LTSTA02-30KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2711	1175.62	GLLGYVPESNK	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]
2712	1175.62	GLLGYVPESNK	
2714	1175.62	GLLGYVPESNK	
3059	2134.01	YVFPQMYFSPELQAATDK	
3063	2134.01	YVFPQMYFSPELQAATDK	
3131	1729.95	KHDVILGLFLFGTDR	
3132	1729.95	KHDVILGLFLFGTDR	
3134	1729.95	KHDVILGLFLFGTDR	
3135	1729.95	KHDVILGLFLFGTDR	
3207	1601.86	HDVILGLFLFGTDR	
3210	1601.86	HDVILGLFLFGTDR	
2774	1667.70	NSWGEDWGENGYVR	>gi|87246235|gb|ABD35294.1| cysteine proteinase [Leishmania tropica]
2777	1667.70	NSWGEDWGENGYVR	
BOTTOMLEYR_XLTSTA028-30KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2714	1175.62	GLLGYVPESNK	>gi|157870820|ref|XP_001683960.1| hypothetical protein [Leishmania major strain Friedlin]ı gi|68127027|emb|CAJ05502.1| hypothetical protein, conserved [Leishmania major strain Friedlin]
2717	1175.62	GLLGYVPESNK	
2720	1175.62	GLLGYVPESNK	

2724	1175.62	GLLGYVPESNK	
3039	2134.01	YVFPQMYFSPELQAATDK	
3110	1729.95	KHDVILGLFLFGTDR	
3111	1729.95	KHDVILGLFLFGTDR	
3113	1729.95	KHDVILGLFLFGTDR	
3114	1729.95	KHDVILGLFLFGTDR	
3182	1601.86	HDVILGLFLFGTDR	
2777	1667.70	NSWGEDWGENGYVR	>gi|87246235|gb|ABD35294.1| cysteine proteinase [Leishmania tropica]
2825	1190.63	TGEVPYWVIK	
BOTTOMLEYR_LTSTA02-58KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2380	1087.53	GSDELNAVQR	>gi|157875225|ref|XP_001686015.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strain Friedlin]ı gi|68129088|emb|CAJ06700.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strai
2384	1087.53	GSDELNAVQR	
2411	1206.63	LTTHAHSVWR	
2570	1249.63	QSGDAVIAFSQK	
2587	978.57	KLETIFTK	
2636	1362.68	ATGETLNPEYLR	
2641	1362.68	ATGETLNPEYLR	
2645	1362.68	ATGETLNPEYLR	
2656	1216.56	IWHFDTDAGR	
2660	1216.56	IWHFDTDAGR	
2740	1217.64	SATALPAEFVGR	
2743	1217.64	SATALPAEFVGR	
2767	1217.64	SATALPAEFVGR	
2770	1217.64	SATALPAEFVGR	
3259	3277.64	SLGLHESQSLFAEFQIGHATPFIDYLTTR	
2504	1059.54	ALLDEAETAK	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
2531	1342.72	AELTTVQQANLR	
2573	1396.71	GLFATIHETGHSK	
2842	1538.80	DAGLEVVAPEAPFPK	
2896	1423.78	LTHLLSLGAWDAK	
2998	1436.73	SNNDFATFLPALK	
3001	1436.73	SNNDFATFLPALK	
3373	2317.27	FAEVVIGHSSAFLEFLTPLLK	
3376	2317.27	FAEVVIGHSSAFLEFLTPLLK	
2389	1150.56	NGAVQVDAYSK	>gi|157863924|ref|XP_001687512.1| trypanothione reductase [Leishmania major]ı gi|68223723|emb|CAJ01955.1| trypanothione reductase [Leishmania major strain Friedlin]
2393	1150.56	NGAVQVDAYSK	
2812	1636.81	TSVDNIYAIGDVTNR	
2815	1636.81	TSVDNIYAIGDVTNR	
3311	3149.54	KSEDPNSDVLETLDTEYILIATGSWPTR	
3353	3021.45	SEDPNSDVLETLDTEYILIATGSWPTR	

3070	2190.11	TIVATGSEPTELPFLPFDEK	>gi|157874347|ref|XP_001685657.1| dihydrolipoamide dehydrogenase [Leishmania major strain Friedlin]ı gi|68128729|emb|CAJ08862.1| dihydrolipoamide dehydrogenase, putative [Leishmania major strain Friedlin]
BOTTOMLEYR_XLTSTA028-56KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2080	1087.53	GSDELNAVQR	>gi|157875225|ref|XP_001686015.1| carboxypeptidase; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strain Friedlin]ı gi|68129088|emb|CAJ06700.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), Family M32 [Leishmania major strai
2371	1206.63	LTTHAHSVWR	
2549	1249.63	QSGDAVIAFSQK	
2605	850.48	LETIFTK	
2620	1362.68	ATGETLNPEYLR	
2623	1362.68	ATGETLNPEYLR	
2630	1345.73	SATALPAEFVGRK	
2635	1216.56	IWHFDTDAGR	
2689	1217.64	SATALPAEFVGR	
2692	1217.64	SATALPAEFVGR	
2816	1107.60	FGFITAPEVK	
2947	983.58	SWLPQLLK	
2491	2134.04	VVSGTNNGSSVTIEVEDKDGK	>gi|157874347|ref|XP_001685657.1| dihydrolipoamide dehydrogenase [Leishmania major strain Friedlin]ı gi|68128729|emb|CAJ08862.1| dihydrolipoamide dehydrogenase, putative [Leishmania major strain Friedlin]
2527	1223.69	ALTDALVKHEK	
2555	1261.63	GEGSFVNPNTIK	
2558	1261.63	GEGSFVNPNTIK	
2875	1196.64	ALTGGVEYLFK	
2879	1196.64	ALTGGVEYLFK	
2935	3252.63	PGHVNYSVIPGVIYTNPEVAQVGETEEQVK	
3010	2318.20	KTIVATGSEPTELPFLPFDEK	
3013	2318.20	KTIVATGSEPTELPFLPFDEK	
3014	2318.20	KTIVATGSEPTELPFLPFDEK	
3062	2190.11	TIVATGSEPTELPFLPFDEK	
2522	1342.72	AELTTVQQANLR	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
2821	1538.80	DAGLEVVAPEAPFPK	
2824	1538.80	DAGLEVVAPEAPFPK	
2926	2384.25	ALLDEAETAKAELTTVQQANLR	
2986	1436.73	SNNDFATFLPALK	
2989	1436.73	SNNDFATFLPALK	
3379	2317.27	FAEVVIGHSSAFLEFLTPLLK	
3383	2317.27	FAEVVIGHSSAFLEFLTPLLK	
2378	1121.57	VVNGINESYK	>gi|157863924|ref|XP_001687512.1| trypanothione reductase [Leishmania major]ı gi|68223723|emb|CAJ01955.1| trypanothione reductase [Leishmania major strain Friedlin]
2381	1121.57	VVNGINESYK	
2783	1636.81	TSVDNIYAIGDVTNR	
3298	3149.54	KSEDPNSDVLETLDTEYILIATGSWPTR	
3301	3149.54	KSEDPNSDVLETLDTEYILIATGSWPTR	
3358	3021.45	SEDPNSDVLETLDTEYILIATGSWPTR	

3361	3021.45	SEDPNSDVLETLDTEYILIATGSWPTR	
BOTTOMLEYR_XLTSTA028-58KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2352	1118.51	NELGEDTVDK	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
2507	1059.54	ALLDEAETAK	
2526	1342.72	AELTTVQQANLR	
2568	1107.56	AYLGLETEGR	
2619	1178.56	TGQMEPIFEK	
2720	1458.72	LDVSEHPFTGMVK	
2735	1543.75	ALIEGTMEAEDIPR	
2738	1543.75	ALIEGTMEAEDIPR	
2808	1538.80	DAGLEVVAPEAPFPK	
2822	1199.69	LSATTPLIWAK	
2825	1199.69	LSATTPLIWAK	
2888	1423.78	LTHLLSLGAWDAK	
2891	1423.78	LTHLLSLGAWDAK	
3027	2384.25	ALLDEAETAKAELTTVQQANLR	
3030	2384.25	ALLDEAETAKAELTTVQQANLR	
3032	1112.62	SWLPELLQK	
3035	1112.62	SWLPELLQK	
3054	1436.73	SNNDFATFLPALK	
3384	2317.27	FAEVVIGHSSAFLEFLTPLLK	
3387	2317.27	FAEVVIGHSSAFLEFLTPLLK	
2390	1150.56	NGAVQVDAYSK	>gi|157863924|ref|XP_001687512.1| trypanothione reductase [Leishmania major]ı gi|68223723|emb|CAJ01955.1| trypanothione reductase [Leishmania major strain Friedlin]
2394	1150.56	NGAVQVDAYSK	
2426	1121.57	VVNGINESYK	
2747	1636.81	TSVDNIYAIGDVTNR	
2750	1636.81	TSVDNIYAIGDVTNR	
3365	3021.45	SEDPNSDVLETLDTEYILIATGSWPTR	
BOTTOMLEYR_XLTSTA030-67KD_072411			
Scan No.	Peptide Mass	Peptide Sequence	Sequence Header
2468	969.56	TLAEVPGRK	>gi|157865648|ref|XP_001681531.1| aminopeptidase; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin]ı gi|68124828|emb|CAJ02694.1| aminopeptidase, putative; metallo-peptidase, Clan MF, Family M17 [Leishmania major strain Friedlin
2471	969.56	TLAEVPGRK	
2679	1110.56	SNVTFTDISK	
2682	1110.56	SNVTFTDISK	
2856	1823.94	KGYTEVMVTALPATTSR	
2891	1695.85	GYTEVMVTALPATTSR	
2661	1342.72	AELTTVQQANLR	>gi|157866094|ref|XP_001681753.1| carboxypeptidase; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain Friedlin]ı gi|68125052|emb|CAJ02401.1| carboxypeptidase, putative; metallo-peptidase, Clan MA(E), family 32 [Leishmania major strain
3504	2317.27	FAEVVIGHSSAFLEFLTPLLK	

Scan No: The scan number of an identified peptide in a LC-MS/MS file. If a peptide has been sequenced more than once, it will have more than one scan numbers.
Peptide Mass: The calculated molecular weight of each peptide based on its amino acid sequence.
Peptide Sequence: The sequence of each peptide identified
Sequence Header: The header of a identified protein present in NR database. It is limited to 300 characters.

For gel samples, since the proteins from same gel bands often have similar mass, the number of peptides sequenced by LC-MS/MS from each protein can be used as an indication for relative abundance of proteins in a sample. For this reason, one peptide sequence may be present several times in the table below if it has more than one LC-MS/MS scans.

Attachment 3

Testing for the Presence of Glycoprotein and Lipoprotein in LtSTA Drug Substance

Leishmania species have lipoproteins on the cell surface which can be additionally modified with different carbohydrate residues. The predominant glycoproteins are believed to be involved in cell adhesion and invasion and include lipophosphoglycans (LPG), proteophosphoglycans (PPG) and secreted glycoproteins. To test for the presence of these compounds, as well as others with carbohydrate components, the drug substance was stained with a glycoprotein staining kit specifically designed for this purpose. LtSTA drug substance is the soluble extract of *Leishmania tropica* promastigotes which have been micro-fluidized and centrifuged to remove non-soluble materials, such as fragments of the cell membrane. The reagents and materials used in this work are shown in the table below.

SDS PAGE sample buffer	Invitrogen # LC2676
SDS PAGE running buffer	Invitrogen # LC2675
Nupage sample reducing agent	Invitrogen # NP0004
See Blue Plus 2 MW marker	Invitrogen # LC5925
Criterion 10-20% Tris-glycine gels, 18 well	Bio-Rad # 3450043
Glycoprotein Staining Kit	ThermoFisher Scientific # 24562
Methyl Alcohol	Spectrum # M1240
Acetic Acid	Spectrum # A1010

Methods:

Leishmania tropica drug substances (XLtSTA027, XLtSTA028, XLtSTA029, XLtSTA030, and XLtSTA031) were diluted to a concentration of 3.2 mg/mL in deionized water. A 22.5 µl sample of each diluted lot was combined with 22.5 µl of SDS PAGE sample buffer bringing the concentration from 3.2 mg/mL to 1.6 mg/ml. 5 µl of Nupage sample reducing agent was then added, bringing the total sample volume to 50 µl and the final sample concentration before loading to 1.44 mg/mL. The diluted samples were mixed well and then heated for 10 min at 100 °C. 30 µl of each sample was loaded onto a Criterion 10-20% Tris-glycine gel (18 wells) in the following order:

Lane	Sample	Amount Loaded (µl)	Amount Loaded (µg)
1	MW marker	10	N/A
3	XLtSTA027	30	43.2
5	XLtSTA028	30	43.2
7	XLtSTA029	30	43.2
9	XLtSTA030	30	43.2
11	XLtSTA031	30	43.2
13	MW marker	10	N/A

15	HRP (+ ctrl)	10	N/A
17	STI (- ctrl)	10	N/A

A positive control Horse Radish Peroxidase (HRP) and a negative control Soybean Trypsin Inhibitor (STI) were also loaded onto the gel. The gel was run at 100 volts for 10 min, 120 volts for 80 min and stained for glycoproteins using a Glycoprotein Staining Kit (ThermoFisher Scientific # 24562) following the protocol provided by the manufacturer

Results:

No distinct bands were observed that demonstrated the presence of glycoproteins. A distinct band at 36 kDa was observed for the positive control. The negative control demonstrated no banding. A faintly visible diffuse pink background smear was present on all samples and controls. In investigating this, it was determined that the diffuse background stain was due to a reaction of the stain with tracer dye and was not indicative of glycoproteins.

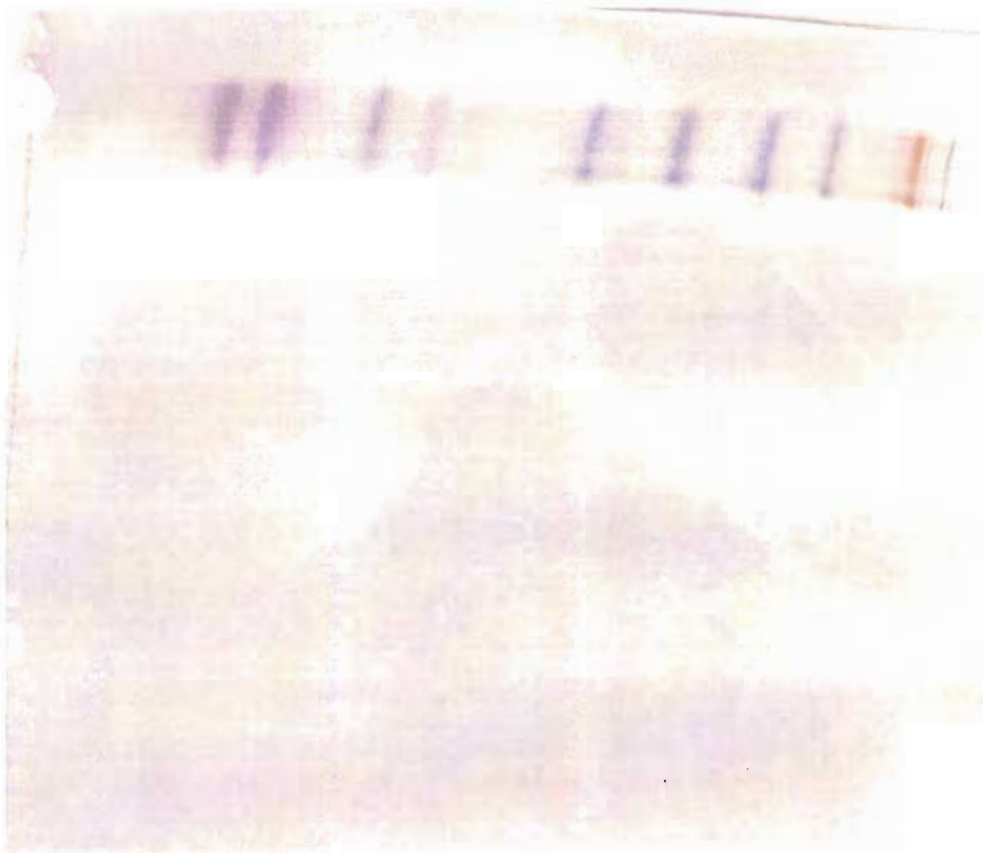
Although glycoproteins are known to be present in *Leishmania tropica*, this material appears to be removed during centrifugation where separation of the soluble cell extract from the insoluble cell materials, including membrane fragment, occurs. Glycoprotein testing of five different lots of drug substance supports this conclusion. Furthermore, the absence of glycoproteins in the concentrated drug substance precludes the presence of glycoproteins in the drug product. In the manufacturing process, the drug substance is diluted in a phosphate buffer with 0.4 % phenol to final product strength of 0.5 mg/mL.

Protein

NuPAGE® Novex® Bis-Tris 4-12% Gel

Approximate Molecular Weights (kDa)

Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS
250	210	188	191
148	105	98	97
98	78	62	64
64	55	49	51
50	45	38	39
36	34	28	28
22	17	17	19
16	16	14	14
6	7	6	n/a
4	4	3	n/a



SBP2 MW Marker

XLtSTA027

XLtSTA028

XLtSTA029

XLtSTA030

XLtSTA031

SBP2 MW Marker

HRP (+ Ctrl)

STI (– Ctrl)

Testing for the Presence of Lipoprotein in LtSTA

Introduction:

LtSTA drug substances was assayed for the presence of lipoproteins using SDS-PAGE (under reducing conditions). The gel was stained using Sudan Black B (Sigma-Aldrich # 199664-25G), which specifically reacts with lipoproteins or other lipid containing substances. LtSTA drug substance is the soluble extract of *Leishmania tropica* promastigotes which have been micro-fluidized and centrifuged to remove non-soluble materials, such as fragments of the cell membrane. The absence of lipoproteins in the drug substance should be indicative of the absence of lipoproteins in the drug product, which is the drug substance diluted to a final Ninhydrin protein concentration of 0.5 mg/mL.

Methods:

Five lots of LtSTA drug substance (XLtSTA027, XLtSTA028, XLtSTA029, XLtSTA030, and XLtSTA031) were diluted to a concentration of 3.2 mg/mL in DI water. 22.5 µl of each diluted lot was combined with 22.5 µl of SDS PAGE Sample Buffer bringing the concentration from 3.2 mg/mL to 1.6 mg/ml. 5 µl of Nupage sample reducing agent was then added bringing the total sample volume to 50 µl and the final sample concentration before loading to 1.44 mg/mL. The diluted samples were mixed well and then heated for 10 min at 100 °C. 30 µl of each drug substance sample (total protein load of 43.2 µg calculated by multiplying load volume by the final sample concentration in µg/mL) was loaded onto a Criterion 10-20% Tris-glycine gel. Additionally, a positive control low density lipoprotein (Sigma # L7914-5MG) and a negative control bovine serum albumin (ThermoScientific # 23210) were also loaded onto the gel. Positive and negative controls were diluted to a final sample concentration of 0.9 mg/mL. 20 µl (18 µg) of each were loaded as assay controls. Nupage sample reducing agent and heat were withheld from both controls. The gel was run at 100 volts for 10 min and then at 120 volts for 80 min. After applying current, the gel was removed from its cassette and stained overnight in 120 mL of Sudan Black B staining solution (formulated per Sigma Aldrich SOP). The gel was de-stained in an acetic acid/acetone buffer over a period of 5 hours.

Results:

Five different lots of drug substance were analyzed by reduced SDS-PAGE and stained with Sudan Black B, which is known to stain specifically for lipoproteins and other lipid containing molecules. Banding was not observed in all 5 lots of drug substance. In contrast, a dark stained band was present in the lane containing the positive control, indicating the presence of lipoprotein, as shown in the attached gel image. Staining of the positive control and the absence of staining with the negative control (bovine serum albumin) demonstrated that the

procedure was performed correctly. Based on these results, LtSTA drug substance does not contain demonstrable amounts of lipoproteins that can be measured using this procedure.

SBP2 MW Marker
XLtSTA027
XLtSTA028
XLtSTA029
XLtSTA030
XLtSTA031
SBP2 MW Marker
LDL (+ Ctrl.)
BSA (- Ctrl.)

Attachment 4

Band	L. major gene	L. major Common Name	L. braziliensi	% identity L.	L. infantum	% identity L.	L. mexicana	% identity L.	T. cruzi	% identity T.	T. brucei	% identity T.	Closest Huma	% identity H.	% identity P.	MW (kDa)
8 KD	ubiquitin															
20 KD	LmjF.36.3990	threonine peptidase	LbrM.35.423	91%	LinJ.36.4180	95%	LmxM.36.399(	94%	Tc00.104705!	78%	Tb11.01.200(	77%	NONE	NA	53%	23.35
	LmjF.32.1830	iron superoxide dismutase	LbrM.32.201	71%	LinJ.32.1920	92%	LmxM.31.183	87%	Tc00.104705!	62%	Tb11.01.755(	68%	chondrial isol	40%	51%	22.98
30 KD	LmjF.25.2010	aldolase	LbrM.25.157	95%	LinJ.25.2090	98%	LmxM.25.201	98%	Tc00.104705!	82%	NONE	NA	NONE	NA	NA	30.30
	LmjF.08.1070	cathepsn L-like protease	LbrM.08.083	74%	LinJ.08.0960	81%	LmxM.08.106	78%	Tc00.104705!	55%	Tb927.6.102(	54%	hepsin precur	< 41%	37%	37.82
	LmjF.08.1020															
	LmjF.08.1050															
56-58 KD	Protease proteins															
	LmjF.33.2540	metalloprotease	LbrM.33.281	84%	LinJ.33.2670	97%	LmxM.32.254	95%	Tc00.104705!	60%	Tb11.02.010(	55%	NONE	NA	NA	57.06
	LmjF.13.0090	metalloprotease	LbrM.13.008	76%	LinJ.13.0090	92%	LmxM.13.009	90%	Tc00.104705!	53%	Tb11.02.010(	51%	NONE	NA	NA	57.04
	LmjF.11.0630	metalloprotease	LbrM.11.041	79%	LinJ.11.0640	90%	LmxM.11.063	83%	Tc00.104705!	39%	Tb11.02.444(	48%	ptidase NPEP	37%	30%	57.16
	Redox proteins															
	LmjF.05.0350	trypanothione reductase	LbrM.05.035	80%	LinJ.05.0350	93%	LmxM.05.035	87%	Tc00.104705!	62%	Tb927.10.10!	64%	e/thioredoxin	< 35%	32%	53.15
	LmjF.32.3310	dihydrolipoamide dehydrogenase	LbrM.32.360	90%	LinJ.32.3510	96%	LmxM.31.331	95%	Tc00.104705!	75%	Tb11.01.847(	75%	dehydrogena	53%	35%	50.58

No Phenol

Band	L. major gene	L. major Common Name
8 KD	ubiquitin	
20 KD	LmjF.36.3990	threonine peptidase
	LmjF.32.1830	iron superoxide dismutase
30 KD	LmjF.25.2010	aldolase
	LmjF.11.0130	NGG1 interacting factor 3-like protein
56 KD	Protease proteins	
	LmjF.33.2540	metalloprotease
	LmjF.13.0090	metalloprotease
	Redox proteins	
	LmjF.05.0350	trypanothione reductase
	LmjF.32.3310	dihydrolipoamide dehydrogenase
67 KD	LmjF.31.0440	cysteine peptidase
	LmjF.11.0630	metalloprotease

Phenol

Band	L. major gene	L. major Common Name
8 KD	ubiquitin	
20 KD	LmjF.36.3990	threonine peptidase
	LmjF.32.1830	iron superoxide dismutase
	LmjF.33.1610	peptidase
30 KD	LmjF.25.2010	aldolase
	LmjF.11.0130	NGG1 interacting factor 3-like protein
56 KD	Protease proteins	
	LmjF.33.2540	metalloprotease
	LmjF.13.0090	metalloprotease
	Redox proteins	
	LmjF.05.0350	trypanothione reductase
	LmjF.32.3310	dihydrolipoamide dehydrogenase
67 KD	LmjF.31.0440	cysteine peptidase
	LmjF.11.0630	metalloprotease
	LmjF.05.0960	metalloprotease

Band	L. major gene	L. major Common Name	Closest Human Gene	% Identity	% Identity P. fal	MW (kDa)
8 KD	ubiquitin					
20 KD	LmjF.36.3990	threonine peptidase	NONE	NA	53%	23.5
	LmjF.32.1830	iron superoxide dismutase	superoxide dismutase [Mn], mitochondrial isoform A precursor [Homo sapiens]	40%	51%	22.9
30 KD	LmjF.25.2010	aldolase	NONE	NA	NA	30.7
	LmjF.08.1060	cathepsn L-like protease	Cathepsin precursors	< 41%	37%	48.7
56-58 KD	Protease proteins					
	LmjF.33.2540	metalloprotease	NONE	NA	NA	54.9
	LmjF.13.0090	metalloprotease	NONE	NA	NA	55.3
	LmjF.11.0630	metalloprotease	probable aminopeptidase NPEPL1 [Homo sapiens]	37%	30%	59.1
	Redox proteins					
	LmjF.05.0350	trypanothione reductase	glutathione/thioredoxin reductase	< 35%	32%	54
	LmjF.32.3310	dihydrolipoamide dehydrogenase	dihydrolipoamide dehydrogenase [Homo sapiens]	53%	35%	52.4

Quarterly Technical Progress Report
July-September (Third Quarter 2010)

Summary:

Please refer to the enclosed Gantt chart for project progress. Work on this project includes:

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1) Phase IIB Clinical Study

A final report of the study was submitted to the FDA and HRPO on January 27,2010. The primary goal of the trial, in addition to safety, was to determine the highest non-sensitizing dose of LtSTA that could be used in a phase III multi-centered study that would provide the necessary information for FDA licensure of the product. The synopsis of the final study report is attached.

2) Production of Additional Lots of LtSTA Products

- Production of Phosphate Diluents, lot numbers 2XPD09091001 & PD09091001.
- Production of Half Schneider's Media, HS08271001 to grow new batches of *L. tropica* for stability lots of LtSTA.
- Production of new Raw Material Lot XLt092110.
- Production of new *Leishmania tropica* Reference Standard, LtLRS02. This reference standard shall be used for potency and identity test controls.

3) Animal Facilities & IACUC:

The semi-annual IACUC meeting was held. New public member, Rebecca Dehbozorgi-Rahimi joined the committee. A brief overview of the functions and responsibilities as an IACUC committee member was explained, protocols reviewed, and the facility was toured. No discrepancies were found.

4) Equipment Calibrations/Repairs/Maintenance:

Calibration of centrifuges, conductivity meter, pressure transmitters, temperature probes, balances and pH meters were all done in this quarter. In addition, the Met-One total air sampler was also calibrated. All equipment was within specification or equipment tolerances.

- The Curtis compressor was serviced; belts changed & air filter replaced.
- The Nikon Inverted Microscope was serviced and cleaned.

5) Validation Testing

A. Preliminary work designed to set assay specifications for the "Validation of the Ninhydrin Assay" (Validation 1074), was initiated. Testing shall be conducted during the fourth quarter.

B. Work progressed on the validation of the Identity Test of LtSTA. The tests are based on SDS-PAGE and ELISA.

C. The Validation VR2013 PQ, 'Validation of Cell Lysis using a 110Y Microfluidizer' was conducted. Three separate validation runs were performed to validate the Microfluidization process incorporated in the production of a crude cellular lysate by demonstrating that the Microfluidizer completely disrupts the *Leishmania tropica* cells. Microphotographs, protein content and cell viability served as indicators of the cell disruption. All acceptance criteria for this validation were met. Validation Report VR2013 was written and must be reviewed and approved.

D. Validation Protocol VP2028 'Validation of Sterile Filtration of Leishmania Skin Test Antigen' was written. The work has been carried out and the testing is in progress. The report will be written once chemistry testing is complete.

E. A validation protocol was started for the validation of the growth curve of L. tropica in 3-Liter aerated Celstirs®. This protocol will be completed and work will be started next quarter.

F. Validation of the Guinea Pig Potency Assay (Validation 2030), has been written and testing initiated.

6) Additional Documentation Processed During the Third Quarter 2010

During the third quarter of 2010 the following documents were revised:

Document	Title	Revision
661-601	Batch Production Record for Leishmania tropica Skin Test Antigen	1
661-605	Batch Production Record for the Production of a Leishmania tropica	1

7) Personnel

Staffing levels for the project are now the principal investigator assisted by one senior technician, a QC analyst, animal care technician, QA manager, regulatory manager and financial manager. The senior technician, QC analyst and animal care technician are full time, whereas the other staff members are part time.

8) Plans for the Fourth Quarter 2010

a. Prepare a response to the FDA letter dated May 2010 and request a Type B meeting with the FDA to discuss the issues mentioned in the letter. In addition, a draft copy of the Phase 3 clinical protocol will be submitted to the FDA for review and comment with Allermed's response to the May 2010 letter.

Requirements of the original contract between USMMDA and Allermed:

2.1 The Contractor shall develop a LST intradermal skin test that meets measurements of efficacy and safety which are required to obtain FDA licensure for human use.

Phase I and phase II testing of the *Leishmania* Skin Test Antigen have been completed. Phase III development of the product is in process.

2.2 The Contractor shall develop a real time shelf life protocol and conduct real time shelf life testing...[and] allow for the option of extending the real time shelf life study for up to five years.

Real time stability studies will be evaluated at refrigerated temperatures. New studies will be initiated on LtSTA at 50 µg/0.1 mL. Previous stability testing has been conducted at 60µg/0.1 mL. Samples shall be stored at 1 - 5°C. Additional testing shall be carried out at higher temperature to determine if LtSTA can be stored and used for short periods of time in areas where refrigeration is not available.

2.3 The Contractor shall prepare the Chemistry, Manufacturing, and Control Data (CMC) section for the new Investigational New Drug Application for the LST.

Complete.

2.4 The Contractor shall prepare and submit to the FDA the Biologics License Application (BLA) required for FDA licensure for human use for the LST.

It is too early in the process to comment on this objective.

2.5 The Contractor shall deliver to the Government quantities of the Leishmania Skin Test (LST) as indicated in section F.

It is too early in the process to comment on this objective.

1. SYNOPSIS

Sponsor: Allermed Laboratories, Inc	BB IND 11822 Protocol LtSTA-08, Rev. 03 and Rev. 03A January 27, 2010	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Antigen		

Title of Study: A Blinded, Placebo Controlled Study Evaluating Safety, False-Positive Reactions and Sensitizing Properties of 15µg, 30µg and 50µg Intracutaneous Doses of Leishmania tropica Skin Test Antigen (LtSTA) In Adult Volunteers Without a History of Exposure to Leishmania spp.

Investigator(s): Donald Brandon, M.D.

Study Center: California Research Foundation

Publication (reference): None

Study Period: Date of first enrollment: 08/01/2008 Date last completed: 09/25/2009	Phase of Development: IIB

Objectives:

1) Primary Objective: Determine if three doses of *Leishmania tropica* Skin Test Antigen (LtSTA) could be safely administered to adult volunteers without previous exposure to *Leishmania* parasites.
2) Secondary Objective: Determine if repeat doses of LtSTA sensitized recipients; determine the highest non-sensitizing dose.

Methodology:

Adult volunteers without known exposure to *Leishmania spp.* were assigned to each of three cohorts. One cohort was skin tested with 0.1mL of 15µg LtSTA; a second cohort was skin tested with 0.1mL of 30µg LtSTA; a third cohort was skin tested with 0.1mL of 50µg LtSTA. Each cohort was given three injections of the assigned strength of LtSTA at 30-day intervals. Skin tests were read 48 hours after the administration of the test article. Induration >5mm was considered a positive DTH response, indicative of sensitization.

Number of Patients: 62 Subjects screened, 50 subjects qualified, 9 subjects dropped, 41 subjects completed the trial.

Diagnosis and Main Criteria for Inclusion:

Volunteers without a history of exposure to *Leishmania spp.*. All volunteers were subjected to a physical examination and laboratory evaluation.

Test Product, Dose and Mode of Administration, Batch Number:

LtSTA is a clear, sterile solution containing the water extractables of *L.tropica* promastigotes. The product is standardized by protein content, stabilized with buffered saline and preserved with 0.4% phenol. The dose was 0.1mL administered intradermally in the forearm. The batch numbers (lots) used in the trial were XLtSTA019 (15µg), XLtSTA018 (30µg), and XLtSTA017 (50µg).

Sponsor: Allermed Laboratories, Inc	BB IND 11822 Protocol LtSTA-08, Rev. 03 and Rev. 03A January 27, 2010	
Name of Finished Product: *Leishmania tropica* Skin Test Antigen (LtSTA)		
Active Ingredient: *Leishmania tropica* Promastigote Antigen		

Duration of Study:
3.5 months for each subject completing the protocol.

Reference Therapy, Dose and Mode of Administration, Batch Number:
See methodology above.

Criteria for Evaluation:
Safety: The safety of LtSTA was based on the absence of local or systemic Adverse Events (AE) associated with its use. Local AE that were monitored included swelling, itching, burning, pain, blistering and necrosis. Systemic AE included body ache, weakness, faintness, dizziness, nausea, flu-like symptoms, difficulty breathing, and post-study abnormal laboratory values.
Efficacy: Efficacy of LtSTA was based on the outcome of the DTH response. Induration < 5mm at 48 hours demonstrated the absence of sensitivity.
Induration > 5mm at 48 hours demonstrated the presence of sensitivity. A 5mm response was considered equivocal.

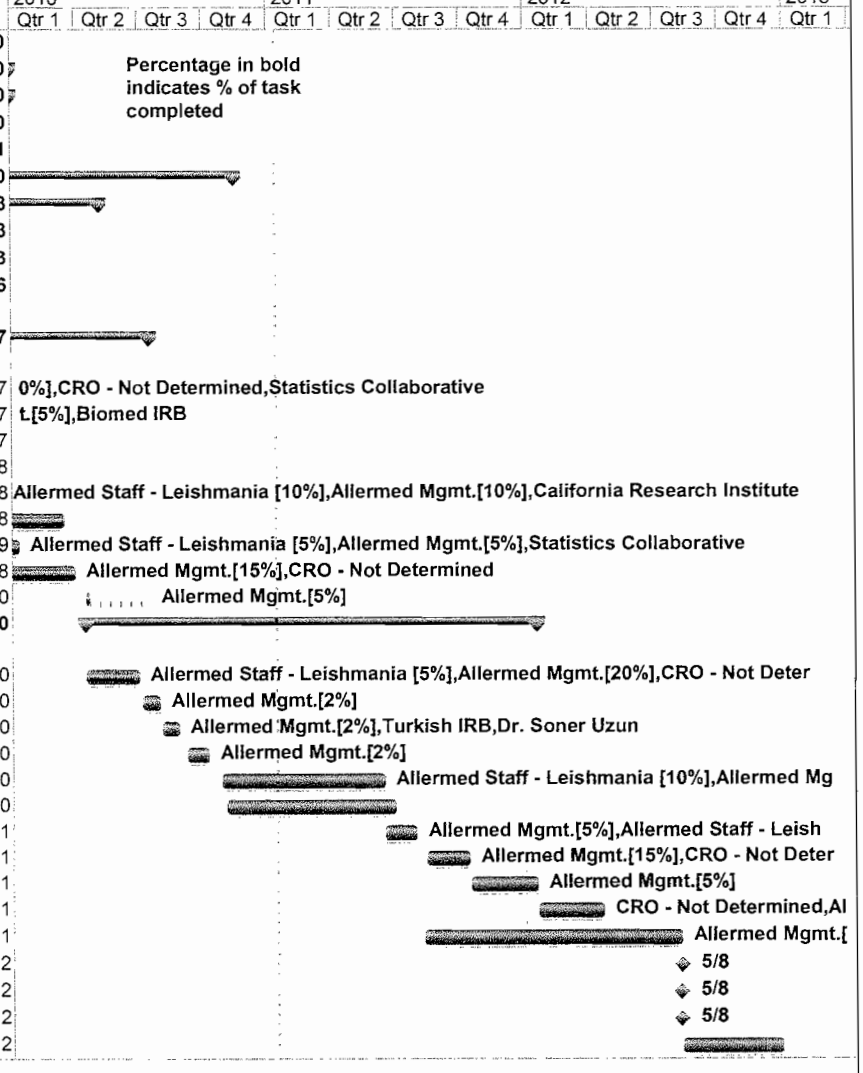
Statistical Methods:
This study was not designed to statistically define the sensitizing properties of LtSTA.

SUMMARY - CONCLUSIONS
Safety Results: Twelve subjects reported AE that implicated LtSTA as the causal agent. These events included local itching, burning and pain at the skin test site. The events occurred within 48 hours after the administration of the test and were mostly mild. Systemic events included body ache, weakness, dizziness and nausea. These events also were mild. One subject had abnormal levels of red blood cells and protein in the post study urine specimens.

Efficacy Results
A 9.0mm induration response to the 30μg dose of LtSTA was observed in one subject on the third skin test. The 50μg dose of LtSTA elicited an 11.5mm induration response on the third skin test in one subject. Induration between 3mm and 5mm was observed to the saline control, placebo control and/or LtSTA in nine subjects. Sensitization to LtSTA was not observed in Leishmania naïve adult volunteers who received two 0.1mL intracutaneous doses of 50μg, 30μg and 15μg at 30 day intervals.

Leishmania Development Project - **Revised**

ID	Task Name	Start
1	**Construction**	**Sun 10/8/00**
5	**Equipment purchase, Installation & Maintenance**	**Fri 10/13/00**
8	**Recruit Personnel**	**Sun 10/1/00**
14	**Develop Organism**	**Fri 12/1/00**
19	**Develop Process**	**Thu 3/1/01**
23	**Validation**	**Fri 12/1/00**
28	**Quality Assurance (compliance, data mgmt, audits & recordkeeping)**	**Tue 5/20/03**
35	**Tasks Associated With the US Army Leishmania Study**	**Mon 10/20/03**
41	**Phase I Safety Study (San Diego, CA: 32 naive subjects)**	**Mon 8/25/03**
53	**Phase II Dose Response Study (Tunisia: 100 subjects split b/t naive, cured and active cutaneous Leishmaniasis)**	**Tue 1/3/06**
64	**Phase IIB Sensitization Study (San Diego, CA: 60 subjects, safety study of established Phase II dose and naïve repeat tests to attempt sensitization)**	**Mon 10/1/07**
65	Write protocol - Phase IIB	Mon 10/1/07
66	Submit to local IRB and obtain approval Phase IIB	Thu 11/1/07
67	Submit protocol to HSRRB and obtain approval Phase IIB	Thu 11/1/07
68	Submit protocol to FDA and obtain approval Phase IIB	Wed 1/2/08
69	Conduct clinical trial Phase IIB	Mon 5/5/08
70	Coordination/Data Management Phase IIB	Mon 5/5/08
71	Perform statistical analysis of study Phase IIB	Tue 9/1/09
72	Write report - Phase IIB	Fri 8/15/08
73	Submit to USAMMDA/WRAIR and FDA for review Phase IIB	Fri 1/15/10
74	**Phase III Efficacy Study (Tunisia: 3 groups, 30 subjects per site. Subjects split b/t naïve, cured and active cutaneous Leishmaniasis)**	**Fri 1/15/10**
75	Write protocol - Phase III	Fri 1/15/10
76	Submit protocol to HSRRB and obtain approval Phase III	Mon 4/5/10
77	Submit to Tunesian IRB and obtain approval Phase III	Mon 5/3/10
78	Submit protocol to FDA and obtain approval Phase III	Mon 6/7/10
79	Conduct clinical trial Phase III	Mon 7/26/10
80	Coordination/Data Management Phase III	Mon 8/2/10
81	Perform statistical analysis of study Phase III	Tue 3/15/11
82	Write report - Phase III	Fri 5/13/11
83	Submit to USAMMDA/WRAIR and FDA for review Phase III	Fri 7/15/11
84	**Prepare BLA**	Mon 10/17/11
85	**Submission & review BLA to FDA**	Mon 5/9/11
86	**BLA Approval by FDA (4QFY09) (Possible BLA fee)**	Tue 5/8/12
87	**Milestone FRP IPR (1QFY10)**	Tue 5/8/12
88	**Product availability for L. tropica (1QFY10)**	Tue 5/8/12
89	**Post FDA licensure - manufacture 50,000 kits**	Wed 5/9/12



Project: Leishmania Skin Test Antigen
Date: Tue 10/12/10

Task | Milestone | External Tasks
Split | Summary | External Milestone
Progress | Project Summary | Deadline

Quarterly Technical Progress Report
October-December (Fourth Quarter 2010)

Summary:

Please refer to the enclosed Gantt chart for project progress. Work on this project includes:

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1) Production of Additional Lots of LtSTA Products

- Lots XLtSTA026, XLtSTA027, XLtSTA028 and XLtSTA029 were microfluidized.
- Lot LtSTA01 was formulated from drug substance lots XLtSTA026 and XLtSTA027, then aseptically filtered and filled.
- Lot LtSTA02 was formulated from drug substance lots XLtSTA028 and XLtSTA029.
- Schneider's media, lot S12011001 and half-Schneider's media were manufactured, sterility tested and released for manufacturing use.

2) Animal Facilities & IACUC:

Animal Care and Use Reports were both submitted to the USDA and the U.S. Army.

3) Equipment Calibrations/Repairs/Maintenance:

1. Biological Safety Cabinets and HEPA filters throughout the cleanroom were serviced as needed and certified.
2. The Microfluidizer was serviced; the motor was disassembled, all gaskets replaced, and flow rate checked and compared to original flow rate.
3. Sanitization of the Deionized Water system was performed and the water tested.

4) Validation Testing

Process Validation

A. Validation Report VR2028 'Validation of Sterile Filtration of Leishmania Skin Test Antigen" was written, reviewed, and approved. The validation work was carried out in the previous quarter.

B. Also in this quarter Validation Report 2026-PQ, Validation of Holding Times of *Leishmania tropica* Skin Test Antigen Drug Product, was written and approved.

C. The Validation VR2013 PQ, 'Validation of Cell Lysis using a 110Y Microfluidizer' was conducted during the third quarter. The validation report was reviewed and approved during the fourth quarter.

D. Validation protocol VP2035 was written to evaluate mixing times of product being formulated at both the ½ strength step as well as final formation of *Leishmania tropica* Skin Test Antigen (LtSTA). The work was performed and the samples analyzed. The report was written and approved.

E. A validation protocol was started for the validation of the growth curve of *L. tropica* in 3-liter aerated Celstirs®. This protocol will be completed and work will be started next quarter.

F. Continued validation of the viability of the cell banks was conducted. One vial from both the Master Cell Bank and the Working Cell Bank were thawed for viability and stability. Both cell banks were observed to be healthy with approximately 80 - 90% viability.

Assay Validation

A. In the previous quarterly report, the Ninhydrin Protein Assay validation for Leishmania was incorrectly reported as 1074. The correct validation number is 2033. The validation was completed this quarter. SOP 916-000 for the Ninhydrin procedure is currently being revised to incorporate improvements based on validation results.

B. Work progressed on the validation of the Identity Test of LtSTA, validation 2034. The tests are based on SDS-PAGE and ELISA. The protocol for the validation has been written.

C. The Orthophosphate Assay, Ascorbic Acid Method (SOP 966-100) is being validated as well. The validation number is VP 2036, and as of this report, testing has been completed. The final report is in process.

D. Validation of the Guinea Pig Potency Assay (Validation 2030), had been written and testing initiated during the third quarter. The following tests have been completed during the fourth quarter, and the data is being analyzed:
   1. Standard vs. Standard using lot LtLRS02 (seven tests of four guinea pigs per test).
   2. Standard vs. Lot 1 using lot XLtSTA016 (seven tests of four guinea pigs per test).
   3. Standard vs. Lot 2 using lot XLtSTA017 (seven tests of four guinea pigs per test).

4. Standard vs. Lot 3 using lot LtSTA01 (seven tests of four guinea pigs per test).

5) Additional Documentation Processed During the Fourth Quarter 2010

During the Fourth quarter of 2010 the following documents were revised:

Document	Title	Revision
SOP 910-	Leishmania Skin Test Antigen Relative Potency Test	1
SOP 910-	Leishmania Skin Test Antigen Relative Potency Test Form	1

Documentation generated in 2010 was reviewed and prepared for end-of-year archival. This includes; equipment logs, temperature charts, test records, and manufacturing records.

6) Personnel

Staffing levels for the project are unchanged. The positions are the principal investigator assisted by one senior technician, a QC analyst, animal care technician, QA manager, regulatory manager and financial manager. The senior technician, QC analyst and animal care technician are full time, whereas the other staff members are part time.

7) Plans for the First Quarter 2011

a. On November 5, a response to the FDA letter dated May 2010 was submitted and a request for a Type B meeting to discuss the issues mentioned in the letter. In addition, a draft copy of the Phase 3 clinical protocol was submitted to the FDA for review and comment with Allermed's response to the May 2010 letter.

Requirements of the original contract between USMMDA and Allermed:

2.1 The Contractor shall develop a LST intradermal skin test that meets measurements of efficacy and safety which are required to obtain FDA licensure for human use.

Phase I and phase II testing of the *Leishmania* Skin Test Antigen have been completed. Phase III development of the product is in process.

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2.3 The Contractor shall prepare the Chemistry, Manufacturing, and Control Data (CMC) section for the new Investigational New Drug Application for the LST.

Complete.

2.4 The Contractor shall prepare and submit to the FDA the Biologics License Application (BLA) required for FDA licensure for human use for the LST.

It is too early in the process to comment on this objective.

2.5 The Contractor shall deliver to the Government quantities of the Leishmania Skin Test (LST) as indicated in section F.

It is too early in the process to comment on this objective.

Leishmania Development Project - **Revised**

ID	Task Name	Start
1	**Construction**	Sun 10/8/00
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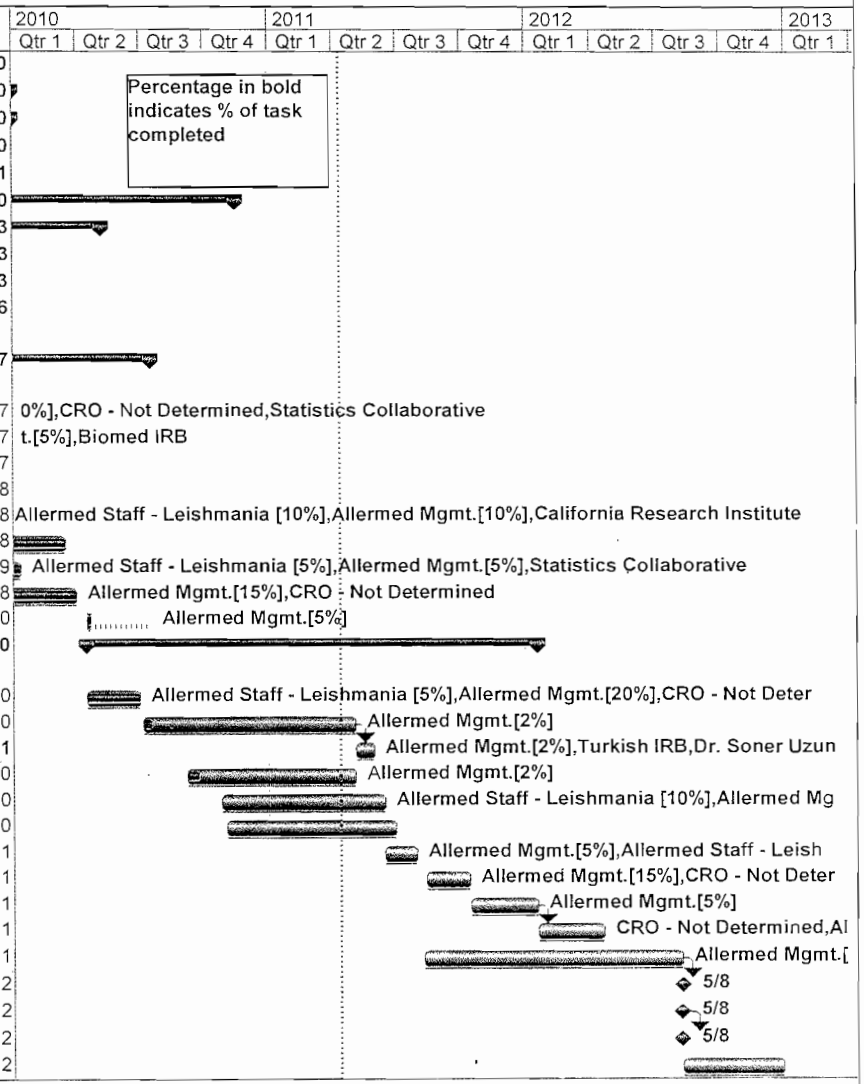
66	Submit to local IRB and obtain approval Phase IIB	Thu 11/1/07
67	Submit protocol to HSRRB and obtain approval Phase IIB	Thu 11/1/07
68	Submit protocol to FDA and obtain approval Phase IIB	Wed 1/2/08
69	Conduct clinical trial Phase IIB	Mon 5/5/08
70	Coordination/Data Management Phase IIB	Mon 5/5/08
71	Perform statistical analysis of study Phase IIB	Tue 9/1/09
72	Write report - Phase IIB	Fri 8/15/08
73	Submit to USAMMDA/WRAIR and FDA for review Phase IIB	Fri 1/15/10
74	**Phase III Efficacy Study (Tunisia: 3 groups, 30 subjects per site. Subjects split b/t naïve, cured and active cutaneous Leishmaniasis)**	Fri 1/15/10
75	Write protocol - Phase III	Fri 1/15/10
76	Submit protocol to HSRRB and obtain approval Phase III	Mon 4/5/10
77	Submit to Tunesian IRB and obtain approval Phase III	Tue 2/1/11
78	Submit protocol to FDA and obtain approval Phase III	Mon 6/7/10
79	Conduct clinical trial Phase III	Mon 7/26/10
80	Coordination/Data Management Phase III	Mon 8/2/10
81	Perform statistical analysis of study Phase III	Tue 3/15/11
82	Write report - Phase III	Fri 5/13/11
83	Submit to USAMMDA/WRAIR and FDA for review Phase III	Fri 7/15/11
84	**Prepare BLA**	Mon 10/17/11
85	**Submission & review BLA to FDA**	Mon 5/9/11
86	**BLA Approval by FDA (4QFY09) (Possible BLA fee)**	Tue 5/8/12
87	**Milestone FRP IPR (1QFY10)**	Tue 5/8/12
88	**Product availability for L. tropica (1QFY10)**	Tue 5/8/12
89	**Post FDA licensure - manufacture 50,000 kits**	Wed 5/9/12



Project: Leishmania Skin Test Antigen
Date: Mon 1/10/11

Task — Split — Progress — Milestone — Summary — Project Summary — External Tasks — External Milestone — Deadline

Confidential

Quarterly Technical Progress Report
January - March (First Quarter 2011)

Summary:

Please refer to the enclosed Gantt chart for project progress. Work on this project includes:

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1) Production of Additional Lots of LtSTA Products

a. The following Solutions were manufactured:

1. Sterile Saline, Lot # SAL01201101
2. 10% Tween 80, Lot # T8002111101
3. Saline with 0.0001% Tween, Lot # SALT02111101
4. Sterile Saline, Lot # SAL02231101
5. Phosphate Diluent, Lot # PD03041101
6. 2X Phosphate Diluent, Lot # 2XPD03041101

b. The following raw material batches were cultured and harvested:

1. XLt011811
2. XLt021511

c. The following batches were Microfluidized, tested for viability, and passed:

1. XLtSTA030
2. XLtSTA031

d. Clinical/stability lot LtSTA01 was labeled and placed at accelerated stability conditions at 23-27°C and 35-40°C (upright and inverted), in addition to refrigerated storage at 2-8°C.

e. Lot LtSTA02 was sterile filtered and labeled for clinical/stability use. Vials were placed on accelerated stability at 23-27°C and 35-40°C (upright and inverted), in addition to refrigerated storage at 2-8°C.

2) Animal Facilities & IACUC:

Final product potency testing was conducted for lots LtSTA01 and LtSTA02. These lots are designated for clinical testing and stability testing. Both lots passed the potency test.

3) Equipment Calibrations/Repairs/Maintenance:

a. An air leak was discovered in the compressor used for Microfluidization. Repairs were made.
b. The Kaye Validator 2000, two SIMS, and the IRTD were sent out for calibration to GE Kaye.

4) Validation Testing

Process Validation

a. A validation protocol was started for the validation of the growth curve of *L. tropica* in 3-liter aerated Celstirs®. This protocol was completed and work will be started next quarter.

Assay Validation

a. SOP 916-000 for the Ninhydrin procedure was revised to incorporate improvements based on validation results.
b. Work was completed on the validation of the Identity Test of LtSTA, validation 2034. The final report will be written.
c. The Orthophosphate Assay, Ascorbic Acid Method (SOP 966-100) validation report was completed (validation 2036).
d. Guinea Pig Potency validation 2030 continued in this quarter. The following tests have been completed and the data analyzed:
    1. Repeat testing of standard vs. Lot 1 (three tests of four guinea pigs per test).
    2. Repeat testing of standard vs. Lot 3 (seven tests of four guinea pigs per test).
    3. All data were submitted to the statistician, analyzed and found to have met the acceptance criteria. From the four (4) sets of 7 tests, acceptance criteria has been calculated for precision (including intermediate precision and repeatability), and Range. This work will be done in the next quarter.

5) Additional Documentation Processed During the First Quarter 2011

During the first quarter of 2011 the following documents were revised:

Document	Title	Revision
301-600	Environmental monitoring of the Leishmania Classified Areas	5
301-600F1	EM of Viable Air Particles in the Leishmania Facility	2
301-600F3	EM of Viable Surface particles in the Leishmania Facility	2
301-600F4	EM Record for Production Activities in the Leishmania Facility	2
401-003	Washing Containers and Utensils in the Bosch Dishwasher #G0032	0 (New)
413-100	Operation of the Centrifuges in the Leishmania Facility	0 (New)
413-100F1	Centrifuge Data Form	0 (New)
644-101	Bubble Point Testing in the Leishmania	0 (New)
644-101F1	Summary of Bubble Point Tests in the Leishmania Facility	0 (New)
660-008	Decontamination procedures in the Leishmania Facility	1
916-000	Total Protein Assay by Ninhydrin	6
918-005	Nonviability Testing of Leishmania Parasite Derived Material	0 (New)
918-005F1	Nonviability Test Result Form	0 (New)
937-101	Bioburden Testing for Leishmania Material	0 (New)
937-101F1	Leishmania Material Bioburden Data Sheet	0 (New)
938-101	Identifying Contamination in Leishmania Cultures	0 (New)
938-101F1	Microbial Contamination in Cultures Result Form	0 (New)

6) Personnel

Staffing levels for the project are unchanged. The positions are the principal investigator assisted by one senior technician, a QC analyst, animal care technician, QA manager, regulatory manager and financial manager. The senior technician, QC analyst and animal care technician are full time, whereas the other staff members are part time.

7) Miscellaneous

a. Experiments have been conducted in this quarter to evaluate optimal storage conditions for the *Leishmania tropica* reference standard.

b. Additionally, we are attempting to further characterize the LtSTA product via SDS-PAGE, looking at glycoprotein and lipoprotein content of the crude cell extract.

c. Twelve (12) additional guinea pigs were ordered and expected to arrive next quarter. These additional guinea pigs will be used for the guinea pig potency validation.

d. A letter was received from FDA in response to the letter dated May 2010. The letter stated that more information will be needed prior to proceeding to phase 3 testing. A teleconference was held with DOD to discuss the letter and how to proceed with this project.

8) Plans for the Second Quarter 2011

Information is being collected to allow Allermed to respond to the FDA letter. We are gathering information on characterization and revising the phase 3 clinical as required.

Requirements of the original contract between USMMDA and Allermed:

2.1 The Contractor shall develop a LST intradermal skin test that meets measurements of efficacy and safety which are required to obtain FDA licensure for human use.

Phase I and phase II testing of the *Leishmania* Skin Test Antigen have been completed. Phase III development of the product is in process.

2.2 The Contractor shall develop a real time shelf life protocol and conduct real time shelf life testing...[and] allow for the option of extending the real time shelf life study for up to five years.

Real time stability studies will be evaluated at refrigerated temperatures. New studies have been initiated on LtSTA at 50 µg/0.1 mL. Previous stability testing has been conducted at 60µg/0.1 mL. Samples shall be stored at 1 - 5°C. Additional testing shall be carried out at higher temperature (23-27°C and 30-35°C) to determine if LtSTA can be stored and used for short periods of time in areas where refrigeration is not available.

2.3 The Contractor shall prepare the Chemistry, Manufacturing, and Control Data (CMC) section for the new Investigational New Drug Application for the LST.

Complete.

2.4 The Contractor shall prepare and submit to the FDA the Biologics License Application (BLA) required for FDA licensure for human use for the LST.

It is too early in the process to comment on this objective.

2.5 The Contractor shall deliver to the Government quantities of the Leishmania Skin Test (LST) as indicated in section F.

It is too early in the process to comment on this objective.

Leishmania Development Project - **Revised**

ID	Task Name	Start	Finish
1	**Construction**	Sun 10/8/00	Tue 6/1/04
5	**Equipment purchase, Installation & Maintenance**	Fri 10/13/00	Wed 9/30/09
8	**Recruit Personnel**	Sun 10/1/00	Wed 9/30/09
14	**Develop Organism**	Fri 12/1/00	Fri 12/20/02
19	**Develop Process**	Thu 3/1/01	Tue 7/21/09
23	**Validation**	Fri 12/1/00	Mon 8/16/10
28	**Quality Assurance (compliance, data mgmt, audits & recordkeeping)**	Tue 5/20/03	Fri 2/5/10
35	**Tasks Associated With the US Army Leishmania Study**	Mon 10/20/03	Fri 3/31/06
41	**Phase I Safety Study (San Diego, CA: 32 naive subjects)**	Mon 8/25/03	Mon 6/5/06
53	**Phase II Dose Response Study (Tunisia: 100 subjects split b/t naive, cured and active cutaneous Leishmaniasis)**	Tue 1/3/06	Wed 12/31/08
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68	Submit protocol to FDA and obtain approval Phase IIB	Wed 1/2/08	Fri 3/14/08
69	Conduct clinical trial Phase IIB	Mon 5/5/08	Fri 9/18/09
70	Coordination/Data Management Phase IIB	Mon 5/5/08	Tue 12/15/09
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72	Write report - Phase IIB	Fri 8/15/08	Thu 12/31/09
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74	**Phase III Efficacy Study (Tunisia: 3 groups, 30 subjects per site. Subjects split b/t naïve, cured and active cutaneous Leishmaniasis)**	Fri 1/15/10	Thu 4/4/13
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76	Submit protocol to HSRRB and obtain approval Phase III	Mon 4/5/10	Mon 5/2/11
77	Submit to Tunesian IRB and obtain approval Phase III	Mon 5/2/11	Fri 5/27/11
78	Submit protocol to FDA and obtain approval Phase III	Mon 6/7/10	Mon 8/8/11
79	Conduct clinical trial Phase III	Mon 8/15/11	Tue 8/14/12
80	Coordination/Data Management Phase III	Mon 8/15/11	Fri 11/9/12
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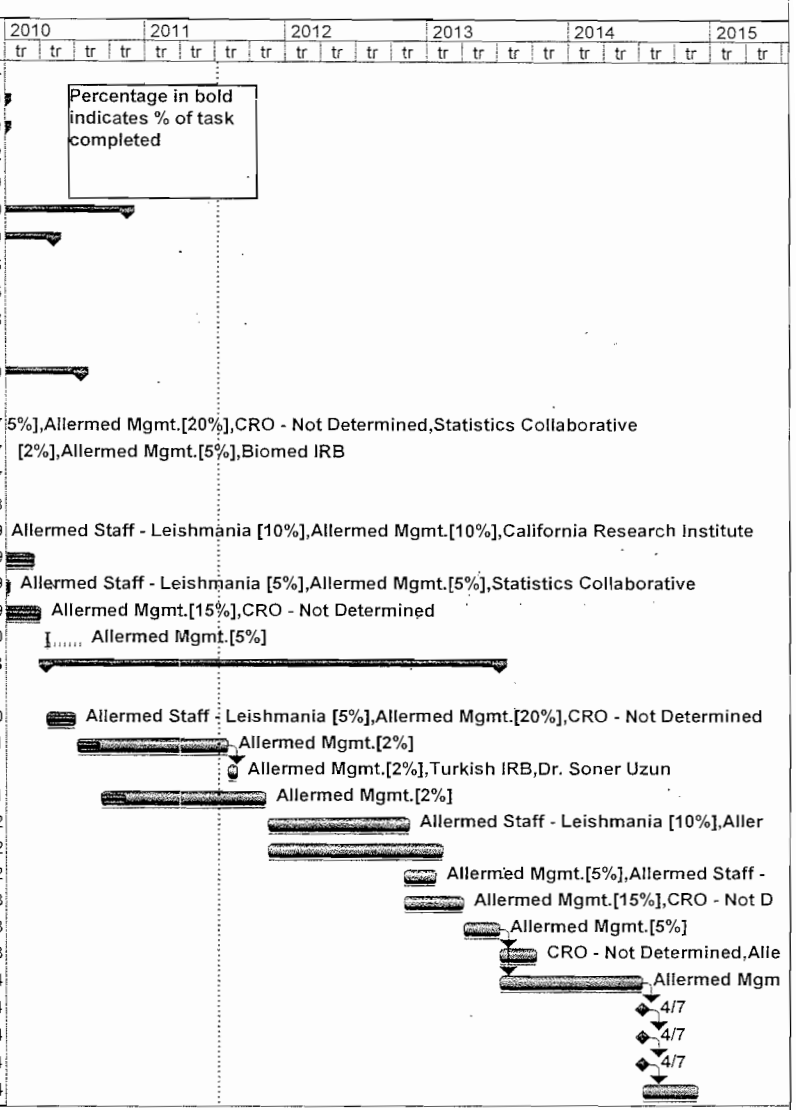
89	**Post FDA licensure - manufacture 50,000 kits**	Mon 4/7/14	Wed 8/27/14



Project: Leishmania Skin Test Antigen
Date: Thu 4/7/11

Task — Milestone — External Tasks
Split — Summary — External Milestone
Progress — Project Summary — Deadline

Quarterly Technical Progress Report
April - June (Second Quarter 2011)

Summary:

Please refer to the enclosed Gantt chart for project progress. Work on this project includes:

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1) Production of LtSTA Products

Drug substance lot numbers XLtSTA030 and XLtSTA031 were combined into LtLRS03, bulk drug product. This lot of drug product will be used to further evaluate the identity of the *Leishmania tropica* product.

Drug product at 500 µg/mL was prepared with and without the addition of the phenol preservative to evaluate the possibility that phenol alters the composition of some constituents of the promastigote lysate. Phenolated and non-phenolated drug product is currently being tested in naive guinea pigs to see if the sensitizing properties of the two preparations differ.

2) Animal Facilities & IACUC

An Institutional Animal Care and Use Committee (IACUC) meeting was held on May 12th. The discussions included the review of the potency protocol and validation, the DEA registration renewal, signing of new acceptance agreements and Program of Veterinary Care agreement. No deficiencies were found in the program.

3) Stability Testing:

a. Lots LtSTA01 and LtSTA02 were tested for 3 month stability at storage temperatures of 2 - 8°C, 23 - 27°C and 35 - 40°C. Testing was conducted on vials stored in the upright and inverted positions.

b. Internal Reference Standard

Storage conditions continue to be evaluated for the *Leishmania tropica* reference standard. Freeze-drying experiments were performed at the final product strength using mannitol and

buffer and WFI and buffer. The results showed diminished signal to noise ratio on ELISA and diminished guinea pig potency in the mannitol preparation.

4) Equipment Calibrations/Repairs/Maintenance:

a. Cleanroom HEPA's and Biological Safety Cabinets (BSC) were tested by Technical Safety Services and recertified.
b. The low-temperature freezer (-80°C), experienced a Freon leak and was repaired. Materials stored in the freezer were transferred into low temperature freezers loaned to us while the leak was repaired. No product issues were encountered.
c. The DI water system was sanitized. No problems were experienced during the sanitization. The water was tested post-sanitization and was found to be in specification.

5) Validation Testing

Assay Validation

a. **Validation Protocol # VP2030** - Validation Protocol for a Relative Potency Test method to Evaluate the Potency of Leishmania tropica Skin test Antigen (LtSTA) with Respect to a LtSTA Internal Reference Standard (LtLRS)

   The validation of the RPTM was completed in this quarter. The data was sent to the statistician, analyzed, and a report was written. Results indicated that the guinea pig skin test does provide a valid measure of product potency. The report is being reviewed and is expected to be signed in during the third quarter.

b. **Validation Protocol #VP2037** - Conversion of Naïve Guinea Pigs to a Positive Skin Test Using LtSTA (Leishmania tropica Skin Test Antigen) Formulated With and Without Phenol

   The purpose of this protocol was to determine if weekly injections of 0.1 mL LtSTA (*Leishmania tropica* Skin Test Antigen) formulated at 50 µg/0.1mL using phosphate diluent *with* 0.4% phenol or LtSTA formulated at 50 µg/0.1mL *without phenol* can cause sensitization by converting a negative skin test to a positive skin test in naïve guinea pigs. Twelve (12) guinea pigs are being used for this protocol; six (6) used to test the product *with* phenol and six (6) used to test the product *without* phenol. The testing was started this quarter and will be completed next quarter.

c. The Leishmania Identity Test ELISA (SOP 944-102) is being reviewed and we are investigating alternative identity tests.

6) Additional Documentation Processed During the Second Quarter 2011

During the second quarter of 2011 the following documents were revised:

Document	Title	Revision
661-605F1	Batch Production Record for the Production of a *Leishmania tropica* Skin Test Antigen	1

7) **Personnel**

Staffing levels for the project are unchanged. The positions are the principal investigator assisted by one senior technician, a QC analyst, animal care technician, QA manager, regulatory manager and financial manager. The senior technician, QC analyst and animal care technician are full time, whereas the other staff members are part time.

8) **Miscellaneous**

Allermed is working on the response to the FDA letter requesting additional product characterization. Efforts are being made to complete the characterization of the Leishmania tropica Skin Test Antigen (LtSTA) by contracting with ProtTech, Inc. (protein quantitation and identification) and Biologics Process Development, Inc. (protein sequencing and identification).

9) **Plans for the Third Quarter 2011**

Information is being collected to allow Allermed to respond to the FDA letter. We are gathering information on characterization and revising the phase 3 clinical as required.

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Leishmania Development Project - Revised

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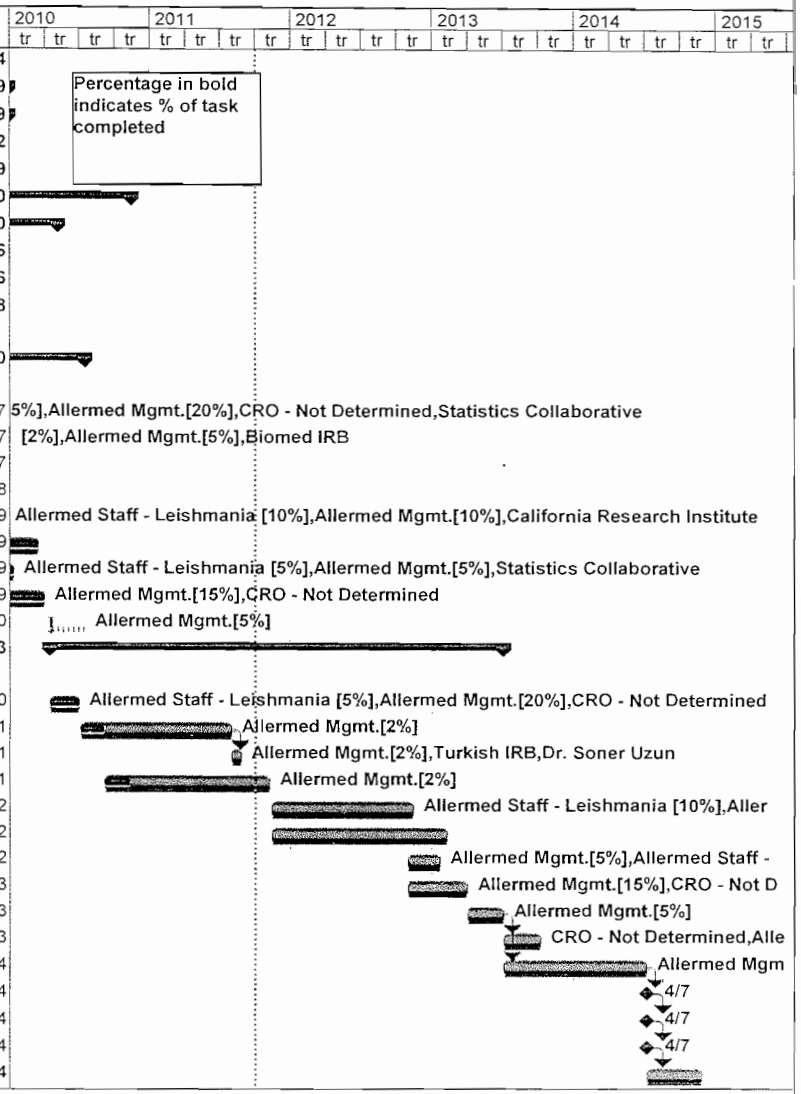

Project: Leishmania Skin Test Antigen
Date: Thu 6/30/11

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